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"Neurotoxicidade central e periférica induzida pela urease majoritária de Canavalia ensiformis em insetos modelo"

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Santos, D. S., Carvalho, E. L., Lima, J. C., Breda, R. V., Oliveira, R. S., **Carrazoni, T.**, Salamoni, S. D., Domingues, M. F., Piovesan, A. R., Boldo, J. T., Assis, D. R., Costa, J. C., Dal Belo, C. A., Pinto, P. M. 2016. "*Bothriurus bonariensis* scorpion venom activates voltage-dependent sodium channels in insect and mammalian nervous systems." *Chemico-Biological Intaractions* 258: 1-9.

II. Artigo publicado 87

Stürmer, G. D., **Carrazoni, T.,** Heberle, M. A., Assis, D. R., Vinadé, L. H., Pereira, A. B., Franco, J. L., Dal Belo, C. A. 2014. "Modulation of dopaminergic neurotransmission induced by sublethal doses of the organophosphate trichlorfon in cockroaches." *Ecotoxicology and Environmental Safety* 109: 56–62.

III. Curriculum Vitae 95

Organização da tese

O corpo da tese é composto por uma introdução geral, objetivos, resultados (artigos), discussão geral, conclusões, referências gerais, anexos.

Os resultados experimentais dessa tese estão apresentados em dois capítulos, cada qual referente a um artigo científico (um já publicado, um como manuscrito).

Capítulo I

"Central and peripheral neurotoxicity induced by the Jack Bean Urease (JBU) in *Nauphoeta cinerea* cockroaches". *Toxicology* 368, 162-171, 2016.

Capítulo II

"Jack bean urease modulates neurotransmitter release on insect neuromuscular junction". Manuscrito.

A metodologia utilizada é descrita em cada um dos artigos.

As referências gerais ao final da tese são relativas à Introdução e Discussão Geral. Referências citadas apenas nos artigos não estão nas referências gerais, apenas no artigo correspondente.

Lista de abreviaturas

JBU: *Jack Bean Urease*

SNC: Sistema Nervoso Central

SNP: Sistema Nervoso Periférico

JNM: Junção Neuromuscular

AP: *Action Potential*

EJP: *Excitatory Junctional Potential*

mEJP: *miniature Excitatory Junctional Potential*

GABA: *Gamma-Aminobutyric Acid*

Glu: Glutamato

OA: Octopamina

ACh: Acetilcolina

AChE: Acetilcolinesterase

Palavras-chave

Canavalia ensiformis; Jack Bean Urease; Neurotoxicidade; *Nauphoeta cinerea*; *Locusta migratoria*; *Drosophila melanogaster*; Neurotransmissão.

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Resumo

Ureases, enzimas que catalisam a hidrólise da ureia formando amônia e dióxido de carbono, são sintetizadas por plantas, fungos e bactérias. A espécie leguminosa *Canavalia ensiformis*, conhecida em inglês como *Jack Bean*, produz diferentes isoformas de ureases, sendo a chamada *Jack Bean Urease* (JBU) a isoforma majoritária. Ureases possuem atividade inseticida já comprovada para diferentes espécies de insetos e um dos seus efeitos no animal é o bloqueio neuromuscular. O objetivo deste trabalho foi compreender o mecanismo de ação responsável pela atividade neurotóxica da JBU e pelo bloqueio neuromuscular em insetos. A injeção de JBU em baratas (*Nauphoeta cinerea*), apesar de não letal, induziu uma inibição da enzima acetilcolinesterase no SNC. A urease, assim como a acetilcolina ou o anticolinérgico neostigmina, também induziu um aumento no ritmo cardíaco das baratas. Insetos tratados com JBU tiveram um aumento no tempo de *grooming*, efeito comportamental este mimetizado com o tratamento por octopamina. Os resultados acima citados indicam um efeito da JBU no SNC do animal, interferindo com a neurotransmissão colinérgica e octopaminérgica e, possivelmente, também GABAérgica.

A atividade de JBU sobre o sistema neuromuscular de insetos também foi estudada através de registros eletrofisiológicos do potencial de ação neural, muscular e de placa motora. Em preparação de músculo tarsal de gafanhoto (*Locusta migratoria*), a urease reduziu a amplitude do potencial de ação evocado; entretanto quando aplicada diretamente no músculo oviductal isolado, a JBU não alterou a resposta contrátil. Em preparações de placa motora de larvas da mosca-da-fruta *Drosophila melanogaster*, a urease aumentou a amplitude dos potenciais excitatórios pós-sinápticos (EJPs) e a frequência dos eventos miniaturas (mEJPs). O efeito da JBU sobre a junção neuromuscular de insetos foi reduzido conforme a concentração de cálcio no meio extracelular também foi reduzida e completamente revertido quando o cálcio foi removido do meio (presença de EGTA e cloreto de Co^{2+}), indicando que a mobilidade de íons de cálcio possui um papel importante na toxicidade induzida por JBU no inseto. Experimentos de imagem de cálcio em gânglios de *N. cinerea* confirmaram um maior influxo de cálcio no neurônio na presença de JBU, indicando que mobilidade do cálcio através da membrana foi alterada. Os resultados obtidos confirmam que a urease induz neurotoxicidade em insetos tanto a nível central como periférico, atuando sobre os gânglios

do cordão nervoso, sobre neurônios aferentes e sobre o terminal pré-sináptico de neurônios motores. A neurotoxicidade induzida por JBU aparenta ser dependente do influxo de íons de cálcio no neurônio.

Abstract

Ureases, enzymes that catalyse the hydrolysis of urea into ammonia and carbon dioxide, are synthesized by plants, fungi and bacteria. The legume *Canavalia ensiformis*, known as jack bean, produces different isoforms of ureases, being the jack bean urease (JBU) its major isoform. Ureases have insecticidal activity against different insect species and one of effects induced by JBU is a neuromuscular blockage. The aim of this work is to further understand the mechanism of action behind this blockage. The injection of JBU into cockroaches (*Nauphoeta cinerea*), despite not lethal, caused inhibition of brain acetylcholinesterase enzyme. Urease, as well as acetylcholine or the anticholinesterase drug neostigmine, induced a positive cardiac chronotropism in cockroaches. Similar to octopamine, JBU induced an increase in the time spent on grooming behaviour. These results indicate that JBU may act on the central nervous system of the animal by interfering with neurotransmission, more specifically on the cholinergic, octopaminergic and, possibly, GABAergic pathways.

The activity of JBU on the neuromuscular junction of insects was studied through electrophysiological recordings. On the tarsus muscle of locust (*Locusta migratoria*), JBU reduced the amplitude of evoked muscle potentials, but did not alter the contractile response when applied to the oviduct muscle isolated. When applied on the end-plate of flies (*Drosophila melanogaster*) JBU led to an increase in the amplitude of Excitatory Junctional Potentials (EJPs), as well as to higher frequency of miniature Excitatory Junctional Potentials (mEJPs). JBU's effect on the neuromuscular junction was reduced when the extracellular calcium concentrations decreased, and it was completely reversed when in calcium free preparations (in the presence of EGTA and Cobalt chloride), indicating that calcium mobility may play an important role in JBU-induced toxicity. Calcium imaging of cockroach ganglions revealed increased calcium influx upon exposition to JBU. These results indicate that JBU induces a central and peripheral neurotoxicity in insects, by interfering on neurotransmitters release or signalling in the ventral nerve cord, in the afferent neurons and in the motor neurons. Moreover, JBU-induced neurotoxicity depends on the influx of calcium ions into the neurons.

1. INTRODUÇÃO

1.1. Ureases de *Canavalia ensiformis*

Ureases são metaloenzimas dependentes de níquel que catalisam a hidrólise da ureia formando amónia e dióxido de carbono, sendo sintetizadas por plantas, fungos e bactérias (Dixon 1975, Follmer 2008). Uma urease amplamente estudada ocorre nas sementes da espécie leguminosa *Canavalia ensiformis*, conhecida popularmente como feijão-de-porco, ou em inglês como *Jack bean*, sendo a sua isoforma majoritária conhecida como *Jack bean urease* (JBU) (Sumner 1926, Pires-Alves et al. 2003, Mulinari et al. 2011). A JBU, assim como outras ureases vegetais, é formada por uma subunidade de 90 kDa (monômero), capaz de associar-se para formar um hexâmero de 540 kDa (Dixon et al. 1975; Takishima et al. 1988; Follmer 2008) (Figura 1). A sequência da JBU é bastante similar a sequência de ureases de outras plantas, mas também à de ureases bacterianas e fúngicas, sugerindo uma origem evolucionária comum (Follmer 2008; Ligabue-Braun et al. 2013). A atividade ureolítica da JBU é altamente dependente de níquel (dois átomos de Ni por monômero) (Dixon et al. 1975); na isoforma de urease conhecida como canatoxina (Carlini et al. 1981), a substituição de um dos níquel por um Zinco reduziu em até 10 x a capacidade ureolítica da enzima (Zerner 1991; Follmer et al. 2002, 2004).

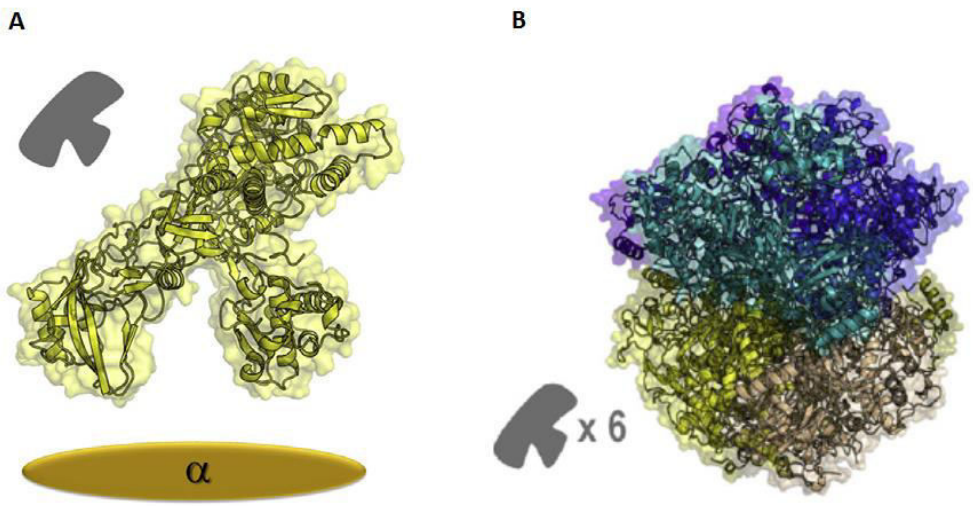

Figura 1. Aspectos estruturais da JBU. (A) Monômero de 90 kDa da urease majoritária de *C. ensiformis* (JBU). **(B)** Associação de monômeros formando um hexâmero de 540 kDa. Figura reproduzida de Carlini & Ligabue-Braun (2016), com modificações.

Entretanto, Ureases, inclusive JBU, também possuem uma ampla gama de atividades biológicas independentes de sua atividade ureolítica (Follmer et al. 2001; Olivera-Severo et al. 2006; Carlini & Polacco 2008; Carlini & Ligabue-Braun 2016). Tais atividades variam desde efeitos como indução de agregação em plaquetas sanguíneas (Carlini et al. 1985; Wassermann et al. 2010), atividade antifúngica para diferentes espécies de fungos filamentosos e leveduras (Becker-Ritt et al. 2007; Postal et al. 2012) até efeitos entomotóxicos para diversas espécies de insetos (Carlini et al. 1997; Carlini & Grossi-de-Sá 2002; Stanisçuaski et al. 2005, 2009; Defferrari et al. 2011; Stanisçuaski & Carlini 2012). Além disso, peptídeos derivados de ureases também demonstram atividade inseticida. Inicialmente descrito para a canatoxina, observou-se que um peptídeo (pepcanatox) obtido a partir de um hidrolisado da proteína com enzimas de larvas do caruncho *Callosobruchus maculatus*, tinha potente atividade inseticida (Carlini et al. 1997; Ferreira-DaSilva et al. 2000). A partir da sequência do pepcanatox, e usando como molde uma terceira isoforma da JBU, a JBURE-II, foi obtido o peptídeo recombinante de 91 aminoácidos, chamado *Jack Bean Urease Toxin* (Jaburetox) (Mulinari et al. 2007), que mostrou possuir atividade entomotóxica contra *D. peruvianus* e *Spodoptera frugiperda* (Mulinari et al. 2007), *Rhodinus prolixus* (Stanisçuaski et al. 2009; Martinelli et al. 2014), *Oncopeltus fasciatus* (Defferrari et al. 2011) e *Phoetalia pallida* (Martinelli et al. 2014). Tanto as duas isoformas de urease (JBU e Canatoxina) quanto o peptídeo recombinante Jaburetox foram extensivamente avaliadas quanto a sua atividade entomotóxica em diferentes espécies de insetos (Tabela 1) (Carlini et al. 1997; Carlini & Grossi-de-Sá 2002; Follmer et al. 2004; Stanisçuaski et al. 2005; Mulinari et al. 2007; Tomazetto et al. 2007; Piovesan et al. 2008; Stanisçuaski et al. 2009, 2010; Defferrari et al. 2011, 2014; Martinelli et al. 2014; Galvani et al. 2015). Acredita-se que a atividade entomotóxica de JBU e Canatoxina possa contribuir para a resistência de *C. ensiformis* contra predação por insetos (Follmer 2004, 2008; Defferrari et al. 2014).

Insect species	Stage or tissue	Type of assay	Toxin	Effect	Inhibited by
Manduca sexta	Larvae	Feeding	CNTX	None	
Schistocerca americana	Larvae	Feeding	CNTX	None	
Drosophila melanogaster	Adults	Feeding	CNTX	None	
Aedes aegypti	Adults	Feeding	CNTX	None	
Callosobruchus maculatus	Larvae	Feeding	CNTX	Lethality	
Nezara viridula	Larvae	Feeding	CNTX	Lethality	
Dysdercus peruvianus	Larvae	Feeding, injection	CNTX JBU	Development delay, lethality	
	Larvae	Feeding, injection	JBTX	Lethality	
Spodoptera frugiperda	Larvae	Feeding	JBTX	Lethality	
Blatella germanica	Larvae	Feeding	JBTX	Lethality	
Oncopeltus fasciatus	Larvae	Feeding, injection	JBU	Lethality	
	Larvae	Feeding, injection	JBTX	Lethality	
Rhodnius prolixus	Larvae	Feeding, injection	CNTX	Development delay, lethality	Cathepsin inhibitors
	Larvae	Injection	JBU	Lethality	Knock-down of a PLA2
	Larvae	Feeding, injection	JBTX	Lethality	
	Malpighian tubules	In vitro	JBU	Inhibition of 5-HT induced secretion -Effect mimicked by PGE2	Dexamethasone, Ca^{2+} chelation or blockers
	Malpighian tubules	In vitro	JBTX	Inhibition of 5-HT induced secretion	
	Anterior midgut	In vitro	JBU	Increase in frequency of 5-HT induced contractions, inhibition of water transport – Effect mimicked by PGE2, increase in tissue PGE2 content	Indomethacin, AH6809 (eicosanoid receptor antagonist)
	Hemocytes	In vitro	JBU	Aggregation	Dexamethasone, Indomethacin, Ca^{2+} chelation
Triatoma infestans	Larvae, Adults	Injection	JBTX	Lethality, neurotoxic symptoms, immunolabeling of CNS, Co-immunoprecipitation with CNS proteins, Inhibition of nitric oxide synthase	
Nauphoeta cinerea	Neuro-muscular junction	In vitro	JBTX	Blockade of evoked contractions of coxal muscle	

Quadro 1. Quadro comparativo dos efeitos entomotóxicos induzidos por JBU, Canatoxina ou Jaburetox (JBTX) em diferentes espécies de insetos. Figura reproduzida de Carlini & Ligabue-Braun (2016), com modificações.

1.2. Atividade entomotóxica da *Jack bean urease*

As atividades inseticidas e entomotóxicas da JBU e seus peptídeos derivados tornaram-se objeto de estudo nas últimas décadas, principalmente devido a sua ampla gama de atuação em diferentes espécies de insetos em concentrações baixas (Stanisçuaski & Carlini 2012). As ureases vegetais possuem atividade tóxica para insetos que utilizam enzimas digestivas do tipo catepsinas, como os hemípteros *R. prolixus* (Carlini et al. 1997),

D. peruvianus (Stanisçuaski et al. 2005) e *O. fasciatus* (Defferrari et al. 2011). Por outro lado, esta atividade tóxica não é observada em insetos que utilizam enzimas do tipo tripsinas, como o lepidóptero *Manduca sexta*; o díptero *Drosophila melanogaster* ou o ortóptero *Schistocerca gregaria* (Carlini et al. 1997). Isto indica que presença ou ausência de toxicidade deve-se, em parte, a forma de processamento das ureases no intestino do inseto. Quando animais dependentes de catepsinas ingerem urease, esta é clivada em sítios específicos liberando peptídeos com atividade entomotóxica (Piovesan et al. 2008; Real-Guerra et al. 2013). Entretanto, já foi demonstrado que JBU também possui atividade tóxica sem sofrer hidrólise alguma, sendo letal quando injetada em determinados insetos (Stanisçuaski et al. 2010). *In vitro*, JBU altera a resposta secretória de túbulos de Malpighi de *R. prolixus* e interfere na resposta contrátil do intestino anterior, além de induzir a formação de agregados de hemócitos *in vivo* e *in vitro* (Stanisçuaski & Carlini 2012; Defferrari et al. 2014). Acredita-se que, além de interferências no metabolismo de eicosanoides, um dos mecanismos para os efeitos entomotóxicos da urease possa ser a desregulação da homeostase dos íons de cálcio através da membrana (Stanisçuaski et al. 2009; Defferrari et al. 2014). Em estudos anteriores do grupo foi demostrado que JBU possui a capacidade de induzir a formação de poros em modelos de bicamadas lipídicas, sendo tais poros altamente seletivos a cátions (Piovesan et al. 2014; Micheletto et al. 2016). Um dos efeitos entomotóxicos induzidos por JBU é um bloqueio neuromuscular, observado em baratas da espécie *Nauphoeta cinerea*. Tal efeito poderia ser devido a uma modulação dos sistemas colinérgicos, glutamatérgicos e/ou GABAérgicos, além de uma modulação na neurotransmissão monoaminérgica do animal, a nível de sistema nervoso central e periférico (Carrazoni et al. 2016). Entretanto, o mecanismo pelo qual esta modulação da neurotransmissão ocorre ainda permanece pouco compreendido. Este trabalho visa gerar conhecimento que permita melhor compreender o papel da JBU na modulação da neurotransmissão em insetos, utilizando três diferentes ordens de insetos: baratas da espécie *Nauphoeta cinerea* (ordem Blattodea), gafanhotos da espécie *Locusta migratória* (ordem Orthoptera) e moscas da espécie *Drosophila melanogaster* (ordem Diptera) (Figura 2).

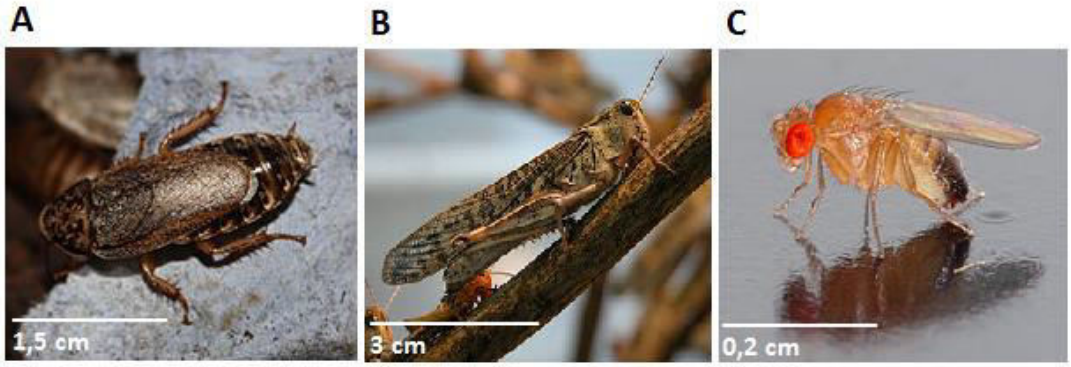

Figura 2. Insetos modelo utilizados neste trabalho. (A) *Nauphoeta cinerea* (Blattodea), **(B)** *Locusta migratoria* (Orthoptera), **(C)** *Drosophila melanogaster* (Diptera).

1.3. Sistema nervoso e neuromuscular de insetos

O sistema nervoso de insetos é formado pelo sistema nervoso central (SNC), composto por gânglios pareados, organizado em forma de um cordão nervoso ventral (Figura 3) e pelo sistema nervoso periférico (SNP), que acompanha o sistema digestivo do animal. Os gânglios anteriores do SNC encontram-se fusionados para formar o cérebro do inseto, composto por três estruturas: protocérebro e deuterocérebro, ambos centros de processamento de informações sensoriais, e o tritocérebro, ligado ao sistema digestivo (Smith 1962; Orborne 1996). Os gânglios que compõem o SNC são formados pelos corpos celulares de neurônios e por células gliais; tais estruturas são conectadas por axônios de neurônios, chamados de conectivos (Figura 4), formando a estrutura do cordão nervoso que corre ao longo do corpo do inseto, localizado ventralmente (Orborne 1996).

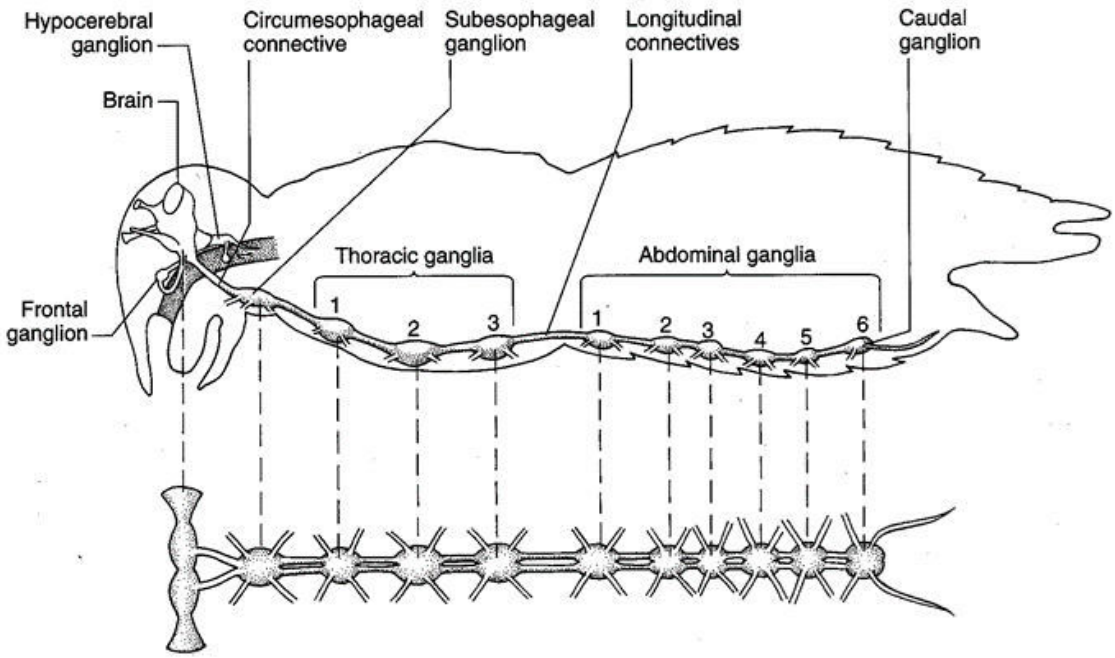

Figura 3. Organização do sistema nervoso central em insetos. Os gânglios da cabeça do animal são fusionados formando a estrutura do cérebro do animal. O restante dos gânglios são pareados e ligados por conectivos de axônios. Figura reproduzida de Romoser & Stoffolano (1998).

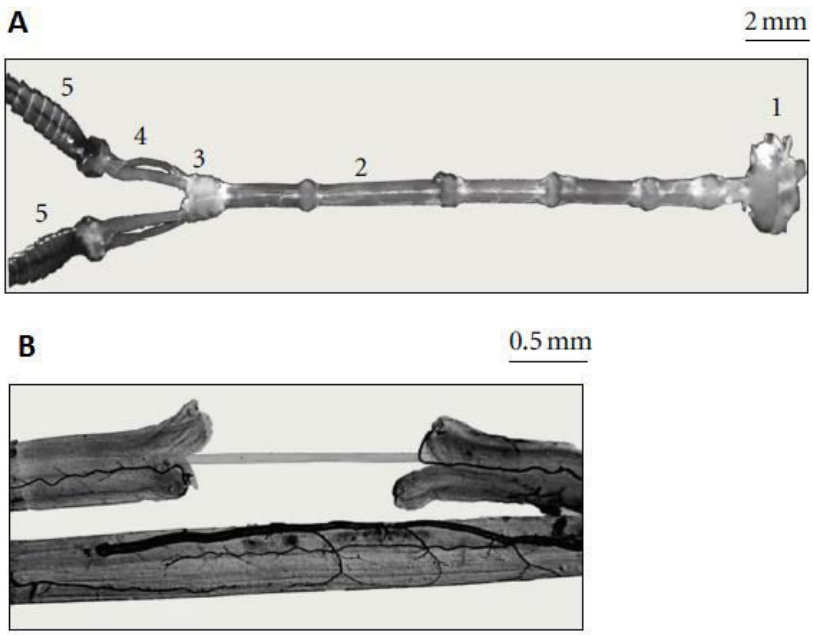

Figura 4. Cordão nervoso ventral de barata da espécies *Periplaneta americana.* (A) Cordão nervoso ventral de *P. americana* mostrando o último gânglio torácico (1), gânglios abdominais e conectivos (2), gânglio cercal (3), nervos cercal (4) e cerci (5). **(B)** Axônio isolado de um conectivo (em cima) e conectivo intacto (abaixo). Diâmetro do axônio ~50 μm. Figura reproduzida de Stankewicz et al. (2012), com modificações.

O sistema de neurotransmissores utilizado pelo SNC de insetos consiste principalmente de acetilcolina e aminas biogênicas, como dopamina, serotonina, adrenalina e octopamina (Clarke & Donnellan 1982; Orchard 1982; Harris & Woodring 1992; Albert & Lingle 1993; Ali et al. 1993; Osborne 1996). Já o sistema neuromuscular, que estabelece a comunicação entre nervo e músculo, utiliza aminoácidos como neurotransmissores, principalmente glutamato (Glu) e ácido γ-Aminobutírico (GABA) (Mathers & Usherwood 1978; Sattelle et al. 1988; Osborne 1996). Em insetos, as sinapses neuromusculares podem variar desde o tipo *endplate* até neurônios motores ramificados (Hamori 1961). Ainda que as propriedades estruturais em muitos aspectos diferem em relação as de sinapses de vertebrados, a organização geral é considerada similar (Couteaux 1958). O músculo de um inseto é inervado por um pequeno grupo de neurônios motores, no máximo oito, que podem evocar diferentes respostas no músculo (Dresden & Nijenhuis 1958). Esses neurônios motores têm origem nos gânglios do cordão nervoso ventral do animal, podem ser excitatórios ou inibitórios (Pringle 1939; Hoyle 1955; Usherwood & Grundfest 1964; Usherwood & Machilli 1966), sendo que um mesmo músculo pode receber inervação das duas classes de neurônios, constituindo um modelo de inervação polineural. Os neurônios motores excitatórios ainda podem ser classificados como rápidos ou lentos, de acordo com o tipo de resposta mecânica que evocam no músculo (Hoyle 1953; Cerf et al. 1959; Usherwood 1962). Em insetos, assim como em vertebrados, a membrana da célula muscular pode ser excitável de forma química ou elétrica. A membrana de células musculares eletro-excitáveis demonstra um tipo de excitação gradativa, com a amplitude do potencial de ação podendo ser gradativa, dependendo da força do estímulo de despolarização. Entretanto, assim como em vertebrados, a excitabilidade da membrana muscular de insetos também possui um período refratário e pode ser inativada após uma longa despolarização (Cerf et al. 1959). Apesar dos neurotransmissores presentes em músculos de vertebrados e insetos serem os mesmos, o sistema de neurotransmissão difere em muitos aspectos. Os neurônios motores de insetos utilizam Glu como neurotransmissor excitatório e GABA como inibitório (Osborne 1996). Os processos que desencadeiam a liberação destes neurotransmissores na sinapse muscular são similares em vertebrados e artrópodes. Um potencial de ação gerado no corpo celular do neurônio motor é propagado através do axônio, por meio do influxo de íons de sódio, e ao atingir o terminal pré-sináptico induz a abertura de canais de cálcio voltagem dependentes, levando a um aumento de íons de cálcio no interior da célula. O aumento da concentração destes íons no

meio intracelular é fundamental para que ocorra o processo de ancoramento, na membrana pré-sináptica, das vesículas contendo o neurotransmissor a ser liberado (Grabnex et al. 1994; Osbourne 1996; Schwarz 2006; Bharadwaj et al. 2010). O neurotransmissor ou neuromodulador liberado na sinapse muscular atua diretamente sobre receptores específicos (ex.: receptor glutamatérgico NMDA no músculo de insetos) para induzir seu efeito (Orchard and Lange 1986). Entretanto, compostos sintéticos, toxinas animais e vegetais podem atuar de forma a modular esse processo de neurotransmissão, induzindo alterações drásticas na neurofisiologia do inseto, podendo inclusive serem letais para o mesmo (Renner et al. 1987; Dulubova et al. 1996; Dal Belo et al. 2005; Quintero-Hernandez et al. 2008; Rattan, 2010; Casida & Durkin 2013; Stürmer et al. 2014). Dessa forma, este trabalho tem por objetivo principal avaliar a interação da entomotoxina JBU, proveniente da leguminosa *C. ensiformis*, com o sistema nervoso (central e periférico) de insetos modelo.

2. OBJETIVO GERAL

O objetivo deste trabalho foi estudar a neurotoxicidade central, periférica e neuromuscular induzida pela urease majoritária de *C. ensiformis* (JBU) em modelos de insetos.

2.1. Objetivos específicos

a. Avaliar a letalidade da JBU em baratas da espécie *Nauphoeta cinerea* e gafanhotos da espécie *Locusta migratoria;*

b. Avaliar a toxidade da JBU sobre a preparação de coração semi-isolado de *N. cinerea*;

c. Avaliar a toxidade da JBU no sistema nervoso central e periférico de *N. cinerea*, através de ensaios bioquímicos para medida da atividade da enzima acetilcolinesterase;

d. Investigar o papel do íon cálcio na toxicidade induzida pela JBU por meio do registro do influxo de cálcio em gânglios metatorácicos de *N. cinerea*;

e. Investigar o mecanismo de ação da JBU sobre o sistema nervoso central de *N. cinerea*, por ensaios e medida da atividade comportamental de *grooming*;

f. Avaliar a neurotoxicidade de JBU em ensaios eletromiográficos em *N. cinerea* e registros eletrofisiológicos em *L. migratoria* e *D. melanogaster*.

3. RESULTADOS

CAPÍTULO I

3.1. Artigo publicado

Thiago Carrazoni, Marines de Avila Heberle, Ana Paula Artusi Perin, Ana Paula Zanatta, Polyana Veloso Rodrigues, Fabiola Duarte Machado dos Santos, Carlos Gabriel Moreira de Almeida, Ricardo Vaz Breda, Douglas Silva dos Santos, Paulo Marcos Pinto, Jaderson Costa da Costa, Celia Regina Carlini, Chárist on André Dal Belo.

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Resumo

Ureases de *Canavalia ensiformis* são inseticidas naturais, entretanto seu modo de ação ainda não é completamente compreendido. Neste trabalho foram investigados os mecanismos envolvidos na neurotoxicidade induzida pela *Jack Bean Urease* (JBU) em baratas da espécie *Nauphoeta cinerea* (Oliver). Para tal, foram utilizadas análises bioquímicas e neurofisiológicas de diferentes órgãos da barata. A injeção de JBU (0.75-6 µg/g de animal) nas baratas, apesar de não ser letal em 24 h, induziu significativa inibição da atividade da enzima acetilcolinesterase no cérebro do animal (60 ± 5 %, $p \leq 0.05$, n = 6). No coração semi-isolado, JBU (1.5 µg/200 µL), acetilcolina (0.3 µg/200 µL) ou neostigmina (0.22 µg/200 µL), induziram um efeito cronotrópico positivo (≈ 25 %) ($p \leq 0.05$, n = 9). A atividade de *grooming* do inseto foi aumentada em 137 (± 7%) com JBU (6 µg/g de animal), de modo similar ao efeito da octopamina (15 µg/g de animal) ($p \leq 0.05$, n = 30). O pré-tratamento do inseto com fentolamida (0.1 µg/g de animal) preveniu o aumento da atividade de *grooming* induzido por JBU ou octopamina. Um bloqueio neuromuscular de 65 ± 9 % foi observado na barata quando o animal foi tratado com JBU (6 µg/g de animal). Este bloqueio foi revertido quando o animal foi pré-tratado com bicuculina (5 µg/g de animal) ($p \leq 0.05$, n = 6). Em ensaios eletrofisiológicos utilizando a perna da barata, a JBU (6 µg/g de animal) induziu uma redução da frequência e um aumento da amplitude dos potenciais neurais espontâneos (1425 ± 52.60 min, 1.102 ± 0.032 mV, $p \leq 0.05$, n = 6, respectivamente). Em suma, estes resultados indicam que a JBU induz efeitos neurotóxicos na barata *N. cinerea* por interferir com o sistema colinérgico, além de causar mudanças comportamentais envolvendo alterações na neurotransmissão octopaminérgica. O bloqueio neuromuscular induzido por JBU sugere um efeito combinado na sinalização por acetilcolina e por GABA.

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Central and peripheral neurotoxicity induced by the Jack Bean Urease (JBU) in *Nauphoeta cinerea* cockroaches

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ABSTRACT

Background: Ureases of *Canavalia ensiformis* are natural insecticides with a still elusive entomotoxic mode of action. We have investigated the mechanisms involved in the neurotoxicity induced by Jack Bean Urease (JBU) in *Nauphoeta cinerea* (Olivier).

Methods: To carry out this study we have employed biochemical and neurophysiological analysis of different cockroach organ systems.

Results and conclusions: The injection of the insects with JBU (0.75–6 μg/g animal), although not lethal within 24 h, caused significant inhibition of the brain acetylcholinesterase activity (60 ± 5%, $p < 0.05$, n = 6). JBU (1.5 μg/200 μL), acetylcholine (0.3 μg/200 μL) or neostigmine (0.22 μg/200 μL), induced a positive cardiac chronotropism (~25%) in the cockroaches ($p < 0.05$, n = 9). JBU (6 μg/g) increased the insects' grooming activity (137 ± 7%), similarly to octopamine (15 μg/g) ($p < 0.05$, n = 30, respectively). Pretreating the insects with phentolamine (0.1 μg/g) prevented the JBU- or octopamine-induced increase of grooming activity. JBU (6 μg/g) caused 65 ± 9% neuromuscular blockade in the cockroaches, an effect prevented by bicuculline (5 μg/g) ($p < 0.05$, n = 6). JBU (6 μg/g) decreased the frequency whilst increasing the amplitude of the spontaneous neural compound action potentials (1425 ± 52.60 min^{-1}, controls 1.102 ± 0.032 mV, $p < 0.05$, n = 6, respectively). Altogether the results indicate that JBU induces behavioral alterations in *Nauphoeta cinerea* cockroaches probably by interfering with the cholinergic neurotransmission. The neuromuscular blocking activity of JBU suggests an interplay between acetylcholine and GABA signaling.

General significance: The search for novel natural molecules with insecticide potential has become a necessity more than an alternative. Understanding the mode of action of candidate molecules is a crucial step towards the development of new bioinsecticides. The present study focused on the neurotoxicity of *Canavalia ensiformis* urease, a natural insecticide, in cockroaches and revealed interferences on the cholinergic, octopaminergic and GABA-ergic pathways as part of its entomotoxic mode of action.

Abbreviations: JBU, Jack Bean Urease; ACh, acetylcholine; CNTX, canatoxin; AChE, acetylcholinesterase; SNCAP, spontaneous neural compound action potentials; DTNB, (5,5′, dithiobis-(2-nitrobenzoic acid); TNB, thionitrobenzoic acid; GABA, gama-aminobutiric acid; GLU, glutamate; EPSPs, excitatory postsynaptic potentials.

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1. Introduction

Throughout the last century, the majority of natural insecticides were replaced by their synthetic counterparts. Amongst the main reasons for the popularization of synthetic insecticides were their low cost, broad spectrum of use covering different insect orders, and longevity in the field. However, although these characteristics appeared initially desirable, they revealed catastrophic consequences for the environment, since these chemical insecticides do not discriminate among insects species, affecting also beneficial ones. In addition, the simplicity of their chemical structures favors insect resistance (Hemingway et al., 2004; Li and Han, 2004; Suzuki and Hama, 1998), which to be overcomed normally requires increasing both dosage and frequency of application further endangering the environment. For these reasons, the search for novel natural molecules with insecticide potential has become a necessity more than an alternative.

Most insecticides exert their effects on insects and other arthropods inducing: (1) neuroexcitation that leads to hyperactivity; (2) spontaneous uncoordinated movements that lead to paralysis due to energy consumption; and (3) neuromuscular failure (Rattan, 2010). Acetylcholine (ACh) is an important neurotransmitter in the evolution of the nervous system (Wächtler, 1988), participating of cell-to-cell communication both in insects and mammals. Comparatively, there are more molecules of ACh in the CNS of insects than in that of a mouse (Sattelle and Breert, 1990). Acetylcholinesterase (AChE), the enzyme responsible for the breakdown of ACh and as such essential for CNS functioning, is one of the main targets of most of the modern insecticides (Leibson and Lifshitz, 2008).

The growing knowledge on the non-enzymatic properties of ureases, plant proteins crucial for the conversion of urea to ammonia, has unveiled their potential as biological insecticides (Carlini and Ligabue-Braun, 2016; Stanisçuaski and Carlini, 2012). Ureases (EC 3.5.1.5, urea amidohydrolase) are nickel-dependent metalloenzymes (Dixon et al., 1975) that catalyze the conversion of urea to two molecules of ammonia and one of carbon dioxide, enhancing the rate of the uncatalyzed hydrolysis by a factor of 8×10^{17} (Callahan et al., 2005). Ureases are widespread in plants, fungi, and bacteria but are not synthesized by animals (Mobley and Hausinger, 1989). *Canavalia ensiformis* displays several urease isoforms: the most abundant one Jack Bean Urease (JBU) (Sumner, 1926), canatoxin (CNTX) (Carlini and Guimarães, 1981; Follmer et al., 2001) and JBURE-II (Mulinari et al., 2011; Pires-Alves et al., 2003). The entomotoxic action of *C. ensiformis* ureases has been shown in different insect models (Stanisçuaski and Carlini, 2012). Afterwards, a recombinant peptide named Jaburetox, derived from the urease isoform JBURE-II of *C. ensiformis*, was produced in *Escherichia coli* and its direct paralyzing effect on the cockroach neuromuscular junction was reported (Martinelli et al., 2014). However, the specific mechanism(s) leading to this biocide activity are still not fully elucidated.

Cockroaches are quite diverse representing more than 4000 species comprising the suborder Dictyoptera. *Nauphoeta cinerea* is an ovoviviparous cockroach of the Blaberidae family. This species is frequently used in biomedical research not only because the easiness of breeding and maintenance but also due to their relative simplicity and suitability for certain experimental procedures (Huber et al., 1990). There are many similarities between the cockroach nervous system and that of other insect species (Huber et al., 1990). In addition their amenability to experimental manipulation and biophysical parallelism with vertebrates make their use very convenient for a number of neurophysiological approaches (Stankiewicz et al., 2012).

In this work we investigated the alterations induced by JBU on the *N. cinerea*'s central and peripheral nervous systems. To accomplish this aim we carried out electromyographic, electrophysiological and behavioral experiments using the cockroach as model.

2. Materials and methods

2.1. Experimental animals

All experiments were performed on adult male *Nauphoeta cinerea* (Olivier) cockroaches (3–4 months after adult molt). The animals were reared in laboratory conditions with controlled temperature (22–25 °C) on a 12 h:12 h L:D cycle. The cockroaches were provided with water and dog chow *ad libitum*.

2.2. Jack Bean Urease (JBU)

Highly purified crystalline urease of *Canavalia ensiformis* (type C3) was obtained from Sigma-Aldrich Brazil. The protein (hexameric molecular mass 545 kDa) crystals were dissolved in 10 mM sodium phosphate buffer and dialysed against deionized water to give 1 μM concentration. Protein solutions were kept at 4 °C and diluted in insect saline to appropriate concentrations before bioassays.

2.3. Reagents and solutions

All chemicals and reagents used were of the highest purity available and were obtained from Sigma-Aldrich, Merck, Roche, Life Technologies or BioRad. Test-solutions were prepared daily by dilution in insect saline immediately before use. The insect saline is a carbonate-buffered solution prepared with the following composition in mM: NaCl, 214; KCl, 3.1; $CaCl_2$, 9; sucrose, 50; HEPES buffer, 5 and pH 7.2 (Stürmer et al., 2014). Except when stated otherwise, all drugs were injected into the abdominal hemocoel, in a 10 μL volume, by means of a Hamilton syringe.

2.4. Lethality assay

The insecticidal assay against adult *Nauphoeta cinerea* was carried out essentially as described by (Kagabu et al., 2007). Various concentrations of JBU dissolved in 10 μL insect saline were injected between the third and the fourth abdominal segments of *N. cinerea*. All the experiments were made in triplicate. Ten insects were used to test each dose. Survival rate was registered 24 h after injection.

2.5. Assay of acetylcholinesterase activity

Acetylcholinesterase activity was evaluated according to Ellman et al. (1961) as modified by Franco et al. (2009) and Stürmer et al. (2014). Briefly, six cockroaches were injected with JBU (1.5, 3 and 6 μg/g body weight), or with trichlorfon (0.03 μg/g), a well known inhibitor of acetylcholinesterase, six hours before the analysis. The animals were anesthetized by chilling for 5–7 min at −20 °C and after cuticle removal, their brains were collected. The material was mixed with 750 μL of potassium phosphate (KPi) buffer pH 7.0. After centrifugation (500 rpm/5 min/4 °C), 50 μL of the supernantant was mixed to 50 mM DTNB (5,5′-dithiobis-(2-nitrobenzoic acid), 500 mL Kpi (pH 8.0) and 2.5 mL acetylthiocholine. The rate of the hydrolysis of acetylthiocholine was measured through the release of thiolcholine, whose free sulphydryl group reacted with DTNB to produce the yellow compound thionitrobenzoic acid (TNB). The reaction was followed at room temperature during 60 s (s) at 412 nm using a UV–vis Spectrophotometer (model Evolution 60S, Thermoscientific, New Hampshire, USA) and analyzed by the software VISION lite (Thermoscientific). The

amount of protein in samples was measured according to Bradford, 1976. The results were expressed as miliunit of AChE per milligram protein (mU/mg protein). One milliunit of AChE was defined as the amount of enzyme able to produce 1 nmol of TNB per min under the specified conditions.

2.6. Semi-isolated cockroach heart preparation

A semi-isolated cockroach heart bioassay was mounted essentially as described by Rodríguez et al. (2012). Briefly adult male cockroaches were anesthetized by chilling (5–7 min) until immobile and placed ventral side up. The lateral margins of the abdomen were cut along each side, and the ventral abdominal body wall was pulled out to show the viscera. After moving the viscera carefully aside the heart was exposed, still contracting while attached to the dorsal body wall. The heart preparations were washed by bathing them in 200 μL of insect saline solution at room temperature (21–24 °C). After 5 min of heart beat stabilization, the treatments were delivered by exchanging the bathing solution. The mean beats.min^{-1} in the first 5 min was taken as a reference. Heart beat frequency was monitored for 30 min under a stereoscopic microscope. Nine cockroaches were used for each group. In the control group, only saline solution was used to bath the heart.

2.7. Behavioral assays

For behavioral studies, animals were placed in an open-field arena (300 × 300 mm, demarcated in 12 zones) with a video camera (Panasonic coupled to a 50 mm Karl-Zeiz lens) mounted overhead as previously described by Stürmer et al. (2014). The camera had a frame-by frame (60 frame/s) device and was connected to a PC (Infoway, ItauTec, Brazil). The insect's activities were recorded during 30 min and the video movies were later analyzed using a HD Writer AE 2.6T system (Panasonic) with variable speed control.

2.7.1. Grooming activity

Cockroaches were examined for grooming behavior (Stürmer et al., 2014) immediatly after injection with JBU (1.5, 3.0 and 6 μg/g). Pretreatment of the cockroaches with phentolamine (0.1 μg/g) or octopamine (15 μg/g) was done by injecting the drugs 10 min prior to JBU (6 μg/g) administration. Grooming behavior was recorded with a camera for later analysis. After treatments the time of continuous grooming in seconds was measured over a 30 min period. Animals had never been placed in the open-field previously, so it was a novel environment in all cases. Testing was performed 2–8 h after the beginning of the light cycle and the room was maintained at 22–25 °C. Control cockroaches were injected only with insect saline.

2.8. Electromyographic recordings

2.8.1. In vivo *cockroach metathoracic coxal-adductor nerve-muscle preparation*

To analyze the effect induced by JBU on insect neuromuscular junctions we used the *in vivo* cockroach metathoracic coxal-adductor muscle preparation essentially as described in Martinelli et al. (2014). Briefly, animals were immobilized by chilling and mounted, ventral side up, in a Lucite holder covered with 1 cm soft rubber that restrained the body and provided a platform to which the metathoracic thigh could be firmly attached using entomologic needles. The left leg was then tied at the medial joint with a dentistry suture line connected to a 1 g force transducer (AVS Instruments, São Carlos, SP, Brazil). The transducer was mounted in a manipulator which allowed adjustment of muscle length. The exoskeleton was removed from over the appropriated thoracic ganglion. Nerve 5, which includes the motor axon to the muscle, was exposed and a bipolar electrode was inserted to provide electrical stimulation. The nerve was stimulated at 0.5 Hz/5 ms, with twice the threshold, during 120 mim. After the insertion of the electrodes, the opening in the exoskeleton was covered with mineral oil to prevent dryness. Twitch tensions were recorded, digitalized and retrieved using a computer based software AQCAD (AVS Instruments, São Carlos, SP, Brazil). Data were further analyzed using the software ANCAD (AVS Instruments, São Carlos, SP, Brazil). The preparations were allowed to stabilize for at least 20 min before injection of drugs into the insect's abdominal hemocoel. Drugs (5 μg/g ACh, or 5 μg/g bicuculline, or 15 μg/g octopamine) were injected 15 min prior the application of JBU (1.5, 3 and 6 μg/g).

2.9. Electrophysiological recordings

2.9.1. In vitro extracellular recordings of spontaneous neural compound action potentials (SNCAP) of the cockroach leg

For the recordings of SNCAP male cockroaches were anesthetized by chilling during 5–7 min and the metathoracic leg was cut as close as possible to the body to ensure that the thigh, femur, tibia, and tarsus remained intact. The leg was then carefully fixed by means of three Ag/AgCl needle electrodes in a petri dish filled with a 10 mm Sylgard® layer. One of the electrodes was connected to the ground connector of the amplifier (Axoclamp 2B, Molecular Devices, USA) and the other to its indifferent (−) connector. The third electrode was placed into the femur as the active recording electrode (+). The signals were recorded at a sampling rate of 1 kHz and digitalized using a digitizer Digidata 1320A (Molecular Devices, USA). The action potentials were visualized, recorded and retrieved for later analysis in the computer-based software Clampex (Molecular Devices, USA), followed by the software WinWCP (John Dempster, University of Strathclyde). Using the described conditions, the preparation could be used for at least one hour without changes in the characteristics of the potentials. The treatments were injected in the leg by means of a Hamilton syringe and the doses were calculated in μg/g based on the weight of the isolated leg.

2.10. Statistical analysis

The results were expressed as mean ± SEM. Each experiment was repeated at least three times. For comparison between means of two different experimental groups the Student "*t*" test was employed. When data from more than two experimental groups were analyzed ANOVA was employed followed by Tukey (all groups were compared with each other) or Dunnet (the groups were compared with a positive control or saline) as *post hoc* tests. All statistical analyses were performed using the Graphpad Prism 6.0 (Software Inc., San Diego, CA). The values were considered significantly different when $p \leq 0.05$.

3. Results

3.1. Entomotoxic activity of Jack Bean Urease (JBU)

To determine the insecticidal activity of JBU, four doses were assayed (0.75, 1.5, 3 and 6 μg/g). After 24 h no lethality was observed. Although not lethal, the animals displayed a notorious grooming activity starting soon after the injection of urease. All insects were lethargic at the end of the 24 h observation period.

3.2. Acetylcholinesterase (AChE) activity in brain homogenates

Analysis of AChE activity of brain homogenates of cockroaches injected with JBU (1.5, 3 and 6 μg/g animal weight) or Trichlorfon (0.03 μg/g) revealed a dose-dependent enzyme inhibition, as compared to controls (insects injected with saline only) (Fig. 1). The control value of AChE activity was 186 ± 3 mU/mg protein/min. At 1.5 μg/g animal weight, JBU induced a significative decrease of the insect brain AChE activity (32.52 ± 3%, n = 6, $p < 0.0001$). At 3 μg JBU/g dose, the AChE activity decreased by 33 ± 3%, n = 6; $p < 0.0001$, and a greater inhibition was seen in brain homogenates of insects injected with JBU at a dose of 6 μg/g animal weight, which produced inhibition of 60 ± 5% (n = 6; $p < 0.0001$) of AChE activity. No difference in the AChE inhibition was seen between the doses of 1.5 and 3 μg JBU/g ($p > 0.05$). As expected Trichlorfon (0.03 μg/g) administration resulted in an AChE inhibition of 77 ± 6% (n = 6; $p < 0.0001$). These results are shown in Fig. 1. As it can be observed the effect of the largest dose of JBU was close to that seen in insects exposed to trichlorphon, a well known AChE inhibitor. Considering the difference in molecular mass of the two compounds, JBU (Mr 540.000) is about 10 times more active than trichlorfon (Mr 257.43) in inhibiting the activity of cockroach brain AChE.

3.3. Effects of JBU on the cockroach's heart rate

The *N. cinerea* semi-isolated heart preparation in the presence of saline had a control value of 76 ± 5 beats/min (n = 9) (Fig. 2A). The addition of JBU to the preparation produced a time-dependent and U-shaped dose-dependent effect. At the lowest dose of JBU (0.75 μg/200 μL) there was an increase of the chronotropic response of the heart, reaching a maximum (99 ± 4 beats/min; n = 9) after 20 min incubation ($p < 0.001$ compared to saline). Starting immediately after addition of 1.5 μg JBU/200 μL, the chronotropic response peaked at 5 min with 97 ± 5 beats/min ($p < 0.001$ compared to saline, n = 9) and lasted the whole 30 min of recording (Fig. 2A). In contrast, when the highest concentration of JBU (3 μg/200 μL) was assayed, no modulation of the chronotropic effect was seen (Fig. 2A). For comparison, ACh (0.3 μg/200 μL) or neostigmine (0.22 μg/200 μL) were assayed in the same preparation, producing a positive modulation of heart beats, with similar results of 92 ± 3 and 90 ± 4, respectively, in 30 min recordings (n = 6), (Fig. 2B).

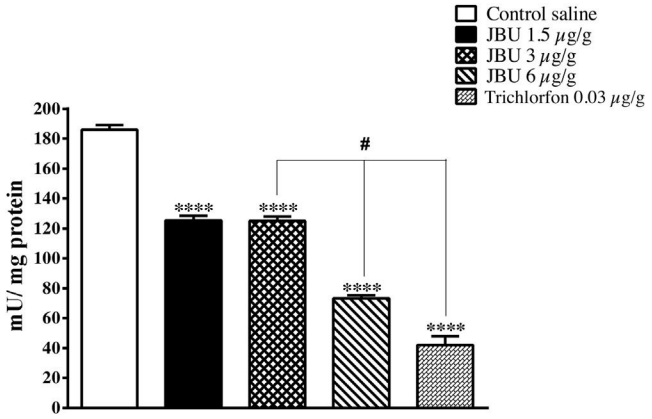

Fig. 1. Acetylcholinesterase inhibition in total cockroach brain homogenates by *in vivo* treatment with JBU.
Cockroaches were injected with JBU (1.5, 3.0 and 6 μg/g; n = 6 for each dose) or trichlorfon (0.03 μg/g) and total brain homogenates were prepared 6 h after the injections. Data were expressed as mU AChE/mg protein of brain homogenate. One mU of AChE is the amount of enzyme hydrolyzing 1 nmol of DTNB per min under the defined conditions (see Methods). The results are expressed as mean ± S.E.M. **** $p < 0.0001$ (n = 6) compared to the control saline by Anova followed by the Dunnett's test; #$p < 0.0001$ comparing each other with Anova followed by the Tukey's test.

3.4. Effects of JBU on the cockroach grooming activity

In saline-injected cockroaches the mean time of continuous grooming was 153 ± 8 s/30 min for the legs and 70 ± 6 s/30 min for the antennae (n = 32, respectively).

Injection of JBU (1.5, 3 and 6 μg/g of animal weight) produced a significant dose-dependent increase in the grooming activity of the leg but had no noticeable effect on the antennae. For 1.5 μg JBU/g animal weight only a tendency of increasing in leg grooming activity was seen (178 ± 24 s/30 min, n = 40; $p > 0.05$, compared to saline) (Fig. 3). At higher doses, JBU (3 and 6 μg/g) induced an evident increase in the leg grooming parameters to 253 ± 33 s/30 min (n = 29) and 363 ± 23 s/30 min (n = 30); $p < 0.002$ and 0.0001 respectively, compared to saline (Fig. 3). The values for antennae grooming were 48 ± 5 s/30 min, 57 ± 7 s/30 min and 81 ± 8 s/30 min, for insects injected with JBU at 1.5, 3 and 6 μg/g animal weight, respectively, (Fig. 3). Only seen at a dose of 1.5 μg/g animal weight, JBU induced a slight reduction of antennal grooming as compared to the control saline ($p < 0.001$), Fig. 3.

3.5. Effects of different pharmacological treatments on JBU-induced grooming activity

Leg grooming activity in insects is thought to be modulated mainly by the neurotransmitter octopamine (Weisel-Eichler et al., 1999). Here, we tested the effects of phentolamine, a selective octopamine receptor blocker, and of octopamine in modulating the behavioral changes induced by JBU. When octopamine (15 μg/g) was injected in the cockroaches, there was an increase in the leg grooming time (134 ± 10%; n = 40; $p < 0.05$ compared to saline; Fig. 4). The treatment of the animals with phentolamine (0.1 μg/g) alone decreased the normal grooming activity to 7.3 ± 2% and 20.3 ± 3.5, for leg and antenna, respectively ($p < 0.01$, n = 30). Administration of phentolamine (0.1 μg/g) 15 min before octopamine injection inhibited the octopamine-induced increase of leg grooming (52 ± 8%; n = 40; $p < 0.05$ compared to the control octopamine; Fig. 4). When phentolamine (0.1 μg/g) was administered 15 min before JBU (6 μg/g) injection, it reverted to control levels the time the insects spent in leg grooming (37 ± 5%; n = 40; $p < 0.05$; Fig. 4).

3.6. Neuromuscular blockade of a cockroach nerve-muscle preparation induced by JBU in vivo

To further analyze the effect of JBU on cockroach nervous system, we used the *in vivo* metathoracic coxal-adductor nerve-muscle preparation. The administration of insect saline alone did not interfere with neuromuscular responses during 120 min recordings (n = 6) (Fig. 5A). JBU (1.5, 3 and 6 μg/g of animal weight) induced a significative time-dependent neuromuscular blockade, which could be seen for the lower doses starting 1 h after injection. For the dose of 6 μg JBU/g body weight the inhibitory effect was noticeable after 30 min, and increased steadly to reach a maximal inhibition of 65 ± 9% (n = 6, $p < 0.05$) after 120 min (Fig. 5A). In the same set of experiments, the treatment of the animals with bicuculline (5 μg/g), a selective blocker of the gama-amino-butyric acid (GABA) receptor, did not affect the twitches' tension during the first 60 min recording, but produced a small (about 15%) although significative blockage after 120 min ($p < 0.0001$, n = 6) (Fig. 6A). When bicuculline (5 μg/g) was injected in the insect 15 min before JBU (6 μg/g), it reduced by ~40% the JBU-induced neuromuscular blockade ($p < 0.05$, compared to the

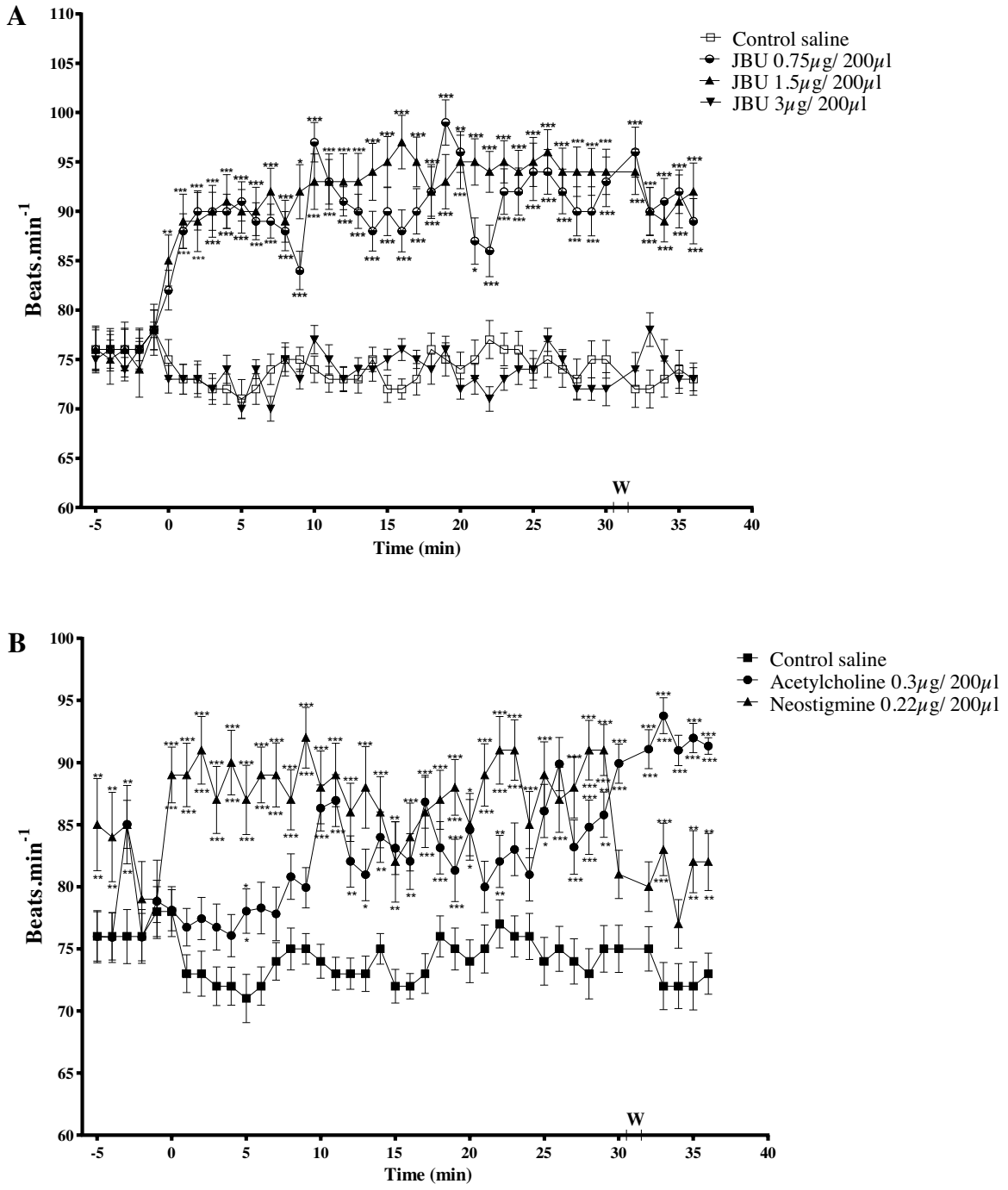

Fig. 2. Effect of different concentrations of Jack Bean Urease (JBU) on *Nauphoeta cinerea* heart rate.
In the graph each point corresponds to the mean ± S.E.M. of the insect heart beats/min relative to the initial state (−5 min), measured during 30 min after exposition to JBU (panel A) or drugs (panel B). Note that JBU (1.5 μg/200 μL) induced a cardio acceleratory activity similar to that induced by ACh (0.3 μg/200 μL) or neostigmine (0.22 μg/200 μL). For data in A and B, statistical analyses were performed by Two-way Anova followed by the Tukey's test. $^{*}p < 0.05$ (n = 9); ** $p < 0.01$; *** $p < 0.001$ (n = 6).

JBU alone) (n = 6, Fig. 6A). For comparison, ACh (5 μg/g) applied alone in the insects induced 52 ± 12% inhibition of the muscle twitches' tension in 120 min (n = 6, $p < 0.05$) (Fig. 6B). Previous application of bicuculline (5 μg/g) 15 min before ACh (5 μg/g) partially prevented (∼40%) the ACh-induced neuromuscular blockade ($p < 0.05$ compared to the ACh alone) (n = 6, Fig. 6B). The administration of octopamine (15 μg/g) alone led to 55 ± 9% neuromuscular blockade of the cockroach neuromuscular junction in 120 min (n = 6, $p < 0.05$) (Fig. 6C). When bicuculline (5 μg/g) was previously applied and followed by octopamine (15 μg/g) there was no prevention of the octopamine-induced neuromuscular blockade (n = 6, $p < 0.05$) (Fig. 6C).

3.7. Effect of JBU on the spontaneous activity of cockroach leg nerve-compound action potentials (SNCAP)

The cockroach leg nerve-compound action potential has a relative high rate of rise when compared to other neuronal models (Table 1). Injection of JBU (6 μg/g) into the cockroach leg caused a significant decrease in the frequency (1425 ± 52.6 min^{-1})

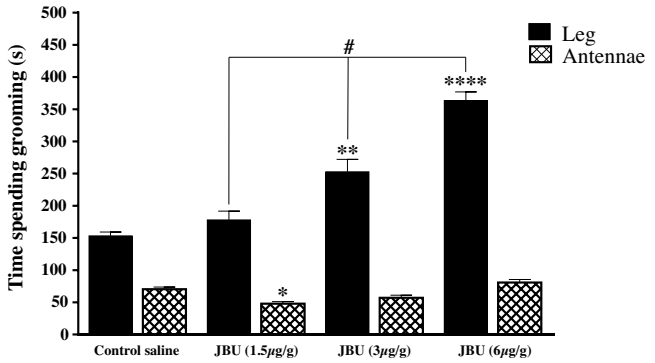

Fig. 3. Increase of grooming behavior induced by JBU in *Nauphoeta cinerea*.
The cockroaches were injected with JBU (1.5, 3.0 and 6 μg/g) and examined for grooming activity immediatly after injection. The grooming activity was recorded during 30 min and the results are expressed as mean ± S.E.M. of the total time of grooms (in s). 30 min^{-1}. The data were analysed by One-way Anova followed by the Dunnett's test.** $p < 0.01$, # $p < 0.001$ **** $p < 0.0001$, n = 28-32, respectively.

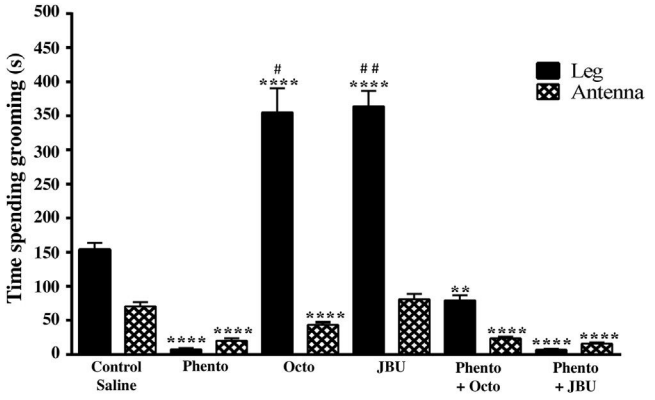

Fig. 4. Effect of different octopaminergic modulators on leg grooming induced by JBU in cockroaches.
The grooming activity was recorded during 30 min immediately after JBU (6 μg/g), octopamine or phentolamine injections. In the case of concomitant treatment with JBU or octopamine (15 μg/g), phentolamine (0.1 μg/g) was injected in the third abdominal segment 15 min before. In the graph, each bar represents the mean ± S.E.M. of the total time of grooms (in s) 30 min^{-1}. Statistical analyses were performed by One-way Anova followed by the Dunnett's test to compare the control saline group with the others. The Student "*t*" test was used to compare the positive control octopamine or JBU with the phentolamine-pretreated groups. **$p < 0.01$; **** $p < 0.0001$; #$p < 0.001$; ##$p < 0.0001$, n = 28-32, respectively.

accompanied by an increase in the mean amplitude (1.102 ± 0.032 mV) of SNCAP (n = 6, $p < 0.05$ respectively) in the 60 min period of observation (Fig. 7A, B). The rise time decreased (0.64 ± 0.044 ms) parallel to the decay time (3.72 ± 2.121 ms, $p < 0.05$ respectively). The treatment with JBU also induced a 9% decrease in the area of the action potentials (Table 1).

4. Discussion

In this work we have characterized the neurotoxic activity induced by the main urease isoform of the *Canavalia ensiformis* plant, the so-called Jack Bean Urease – JBU, in the cockroach *Nauphoeta cinerea*. Aspects related to the cellular and biochemical mechanisms involved in neuromodulation of the insect central and peripheral nervous system by JBU were elucidated in this study.

Although JBU has been proven to be insecticidal to different insects (Stanisçuaski and Carlini, 2012), it displayed no lethality in *Nauphoeta cinerea* cockroaches, at least not by intrabdominal administration in a 24 h observation time. In previous studies, we reported that the lethality of Jack Bean ureases given orally to insects depends on characteristics of their digestive system (Stanisçuaski and Carlini, 2012). At least part of the insecticidal effect is caused by toxic peptides derived from ureases upon cleavage by insect's digestive enzymes. Thus, only insects relying on cathepsin-like enzymes (cysteine and aspartic proteases) *e.g. Callosobruchus maculatus* and *Rhodnius prolixus*, died upon ingestion of ureases, while insects with digestion based on trypsin-like enzymes (serine proteases), such as *Manduca sexta*, *Schistocerca americana*, *Drosophila melanogaster* and *Aedes aegypti*, were not killed. Elpidina and cols (Elpidina et al., 2000) showed that the digestion in *N. cinerea* midgut is carried out with optimal activity at pH 11.5 by three types of serine proteinases and one cysteine proteinase, what could explain the absence of lethality of JBU in this insect model. On the other hand, it is already known that urease-derived peptides do not account for all the entomotoxic properties displayed by these proteins. The whole protein can be found circulating in the hemolymph of insects after feeding (Stanisçuaski et al., 2010). Urease itself was shown to produce entomotoxic effects in the hemipteran *Rhodnius prolixus* that were independent of enzymatic cleavage, such as impairment of diuresis in isolated Malpighiam tubules (Stanisçuaski et al., 2009) or increase in the frequency of crop contraction (Stanisçuaski et al., 2010). Here we have shown that, even in an insect model probably unable to cleave JBU to release its insecticidal peptides, thus not causing lethality, the whole protein induces profound alterations of the cockroach physiology, impacting both its central and peripheric nervous systems.

JBU-induced effects in *N. cinerea*, particularly the positive cardiac chronotropism and behavioral alterations, are consistent with inhibition of the cockroach's brain AChE activity (Stürmer et al., 2014). The precise molecular mechanism for this anti-AChE-like effect of JBU is presently unknown. A number of possibilities can be raised to explain this property of JBU, including but not limited to: (1) a direct interaction of JBU with the AChE molecule leading to its inhibition; (2) an "agonist"-type of interaction of JBU directly with ACh receptors; (3) an interaction of JBU with cell membranes in the vicinity of ACh receptors in a way that leads to their activation; (4) an interaction of JBU with sodium channels coupled to ACh receptors. It is already known that JBU is able to insert itself in lipid bilayers thereby alterating physicochemical parameters of lipid membranes (Piovesan et al., 2014) and that JBU activates sodium channels in a number of systems (unpublished data). Thus, one or more than one of the possibilities raised above could explain the anti-AChE of JBU in the cockroach brain homogenate as well as its other "cholinergic-like" effects.

The increase in leg grooming rather than that of antennae and its antagonism by phentolamine in JBU-treated cockroaches suggests the involvement of the neurotransmitter octopamine in the modulation of the cockroach's behavior (Weisel-Eichler et al., 1999) as triggered by the toxin. The observed inhibition of antennal grooming by phentolamine may be a result of its unspecific antagonistic action upon other monoaminergic receptors (Koons et al., 1983; Tayo, 1979). In insects, although the neural center involved in grooming behavior has not been identified so far, it is known that monoamines such as dopamine and octopamine modulate this behavioral activity (Libersat and Pflueger, 2004). Considering that cholinergic-octopaminergic signaling is a common physiological aspect of the insect CNS (Buhl et al., 2008), it is possible that the anti-AChE-like activity of JBU underlines the alterations in grooming behaviour evoked by the toxin in the cockroaches.

The anti-AChE-like activity of JBU could also account for the increase in the heart rate in our experimental model. In *Periplaneta americana*, the heart rate is determined by a neurogenic pacemaker with cholinergic properties (Wigglesworth, 1972), stimulated by ACh and by AChE inhibitors (Husmark and Ottoson, 1971a,

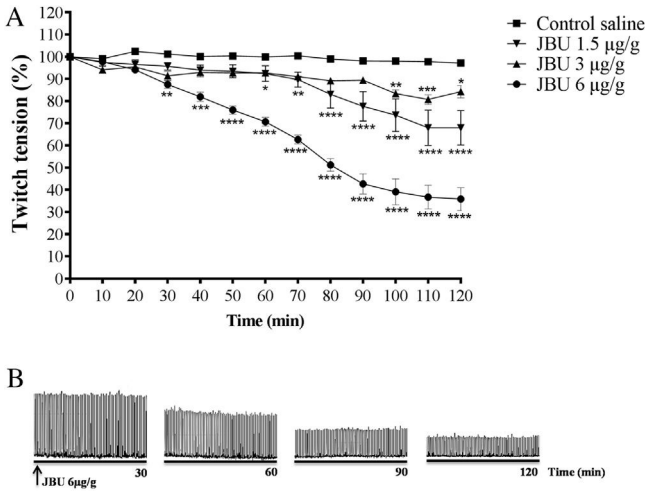

Fig. 5. Neuromuscular blockade induced by Jack Bean Urease (JBU) in *Nauphoeta cinerea* cockroaches.
In the graph (A) each point represents the mean ± S.E.M. of the twitch tension percentage relative to before the treatments. Panel B shows a representative recording of the JBU-induced neuromuscular blockage. Statistical analyses were performed by Two-way Anova followed by the Tukey's test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, n = 6, respectively.

bDahm, 1971). In a previous study, our group has demonstrated the increase of heart rate in *Leurolestes circunvagans* cockroaches by naturally occurring inhibitors of AChE (Rodríguez et al., 2012). The molecular mechanism(s) by which JBU leads to AChE inhibition was not investigated in the present work. Besides an interference of JBU on the cholinergic regulation of *N. cinerea*'s heart, there are other mechanisms that could account for the effects seen and that were not explored here. The cardioacceleratory peptide proctolin (Sliwowska et al., 2001), 5-hydroxytryptamine (5-HT) and, more relevant, octopamine (Tublitz and Truman, 1985) also function as cardioregulatory neurohormones (Miller, 1979). Since octopamine has a biphasic effect over the cardiac rhythm in other insects (Papaefthimiou and Theophilidis, 2011), it is also possible that the cholinergic overstimulation induced by JBU accounts for the modulation of the cockroach cardiac rhythm through an octopaminergic cotransmission.

The electromyographic recordings of cockroaches injected with JBU revealed that the whole protein has a neuromuscular blocking activity. This effect was previously reported for Jaburetox, a urease-derived recombinant peptide that corresponds to about one tenth the size of the whole protein (Martinelli et al., 2014). Several biological properties, but not all of them, are shared between the whole urease molecule and Jaburetox. Particularly relevant to the present data, it has been shown that in nanomolar concentrations both JBU and Jaburetox are able to insert themselves into artificial lipid bilayers creating cation-selective channels (Piovesan et al., 2014). The region of the JBU molecule comprising Jaburetox's sequence is well exposed at the protein's surface, hence it probably mediates most of the interactions of the urease molecule with its targets (Piovesan et al., 2014).

There is a considerable amount of work showing that insect neuromuscular junctions rely on glutamate (GLU) as the main

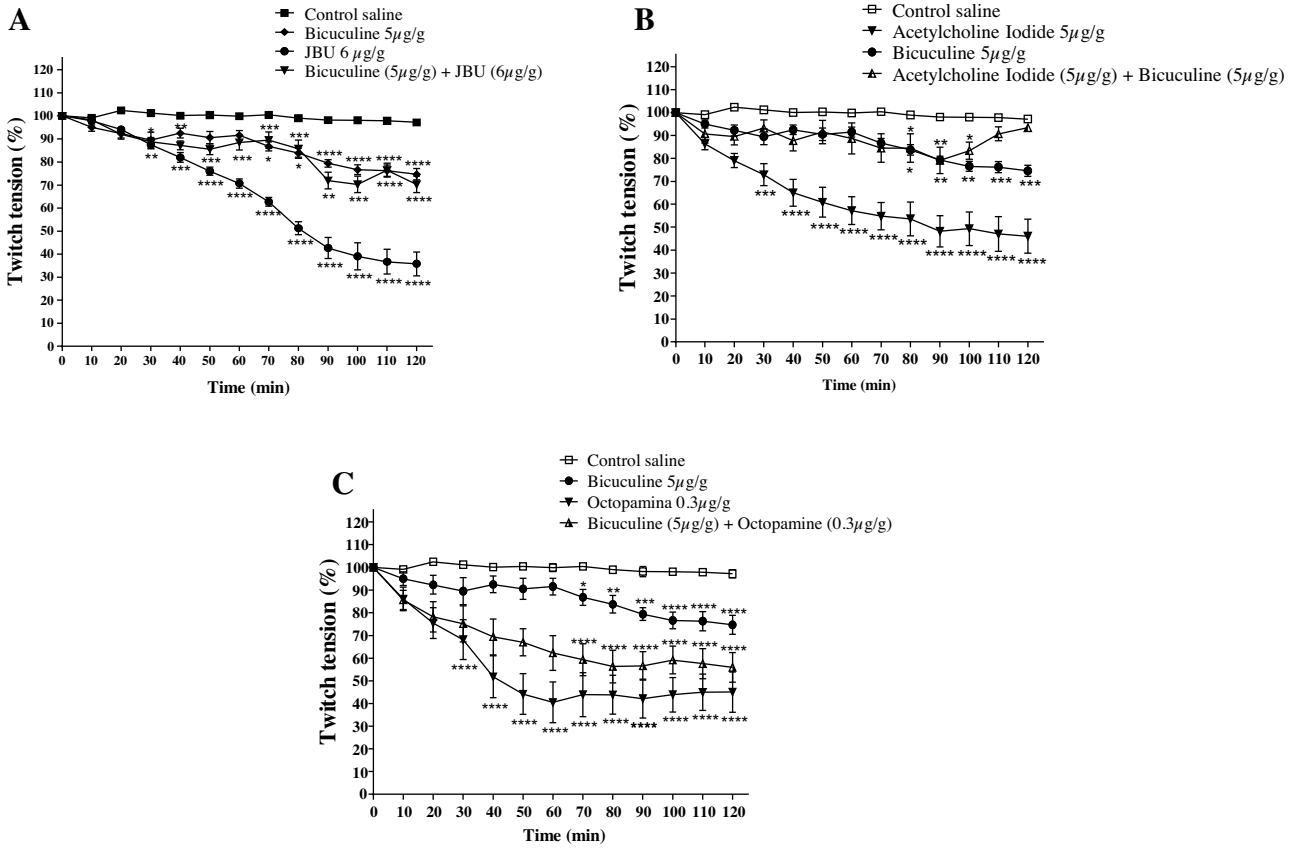

Fig. 6. Neuromuscular blocking activities induced by Jack Bean Urease, octopamine and acetylcholine and its prevention by bicuculline using *in vivo* essays with *Nauphoeta cinerea* cockroaches.
In A, B and C, note the similarity among the neuromuscular inhibitory activity of jack bean urease (6 µg/g), acetylcholine (5 µg/g) and octopamine (15 µg/g). In all cases, the administration of bicuculline (5 µg/g) 15 min before counteracted the neuromuscular blockade. The results are expressed as mean ± S.E.M. The statistical analyses were performed by Two-way Anova followed by the Tukey's test. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, n = 6, respectively.

Table 1
Effects of JBU on cockroach sensorial compound action potentials (SNCAP).

	Frequency (Events.60 min^{-1})	Amplitude Average (mV)	Rise time (ms)	Decay time (ms)	Area (mV.ms)
Control saline	3685 ± 273	0.0599 ± 0.0.19	3.72 ± 0.264	13.866 ± 8.03	0.939 ± 0.1339
JBU 6 μg/g	1425 ± 52.19*	1.102 ± 0.06*	0.64 ± 0.04*	3.72 ± 2.121*	0.724 ± 0.099

The SNCAP traces in Fig. 7 were analysed to extract their frequency (events. 60 min^{-1}), average amplitude (mV), rise and decay time (ms) and average area under the traces (mV.ms). Note that treatment with JBU induced a decrease in the frequency and an increase in the amplitude of the events in 60 min recordings. The rise and decay times and total area of the events were also reduced in JBU-treated preparation. Data are means ± S.E.M. of n = 9 replicates. The means of each parameter were compared using Student "*t*" test.
* $p < 0.05$.

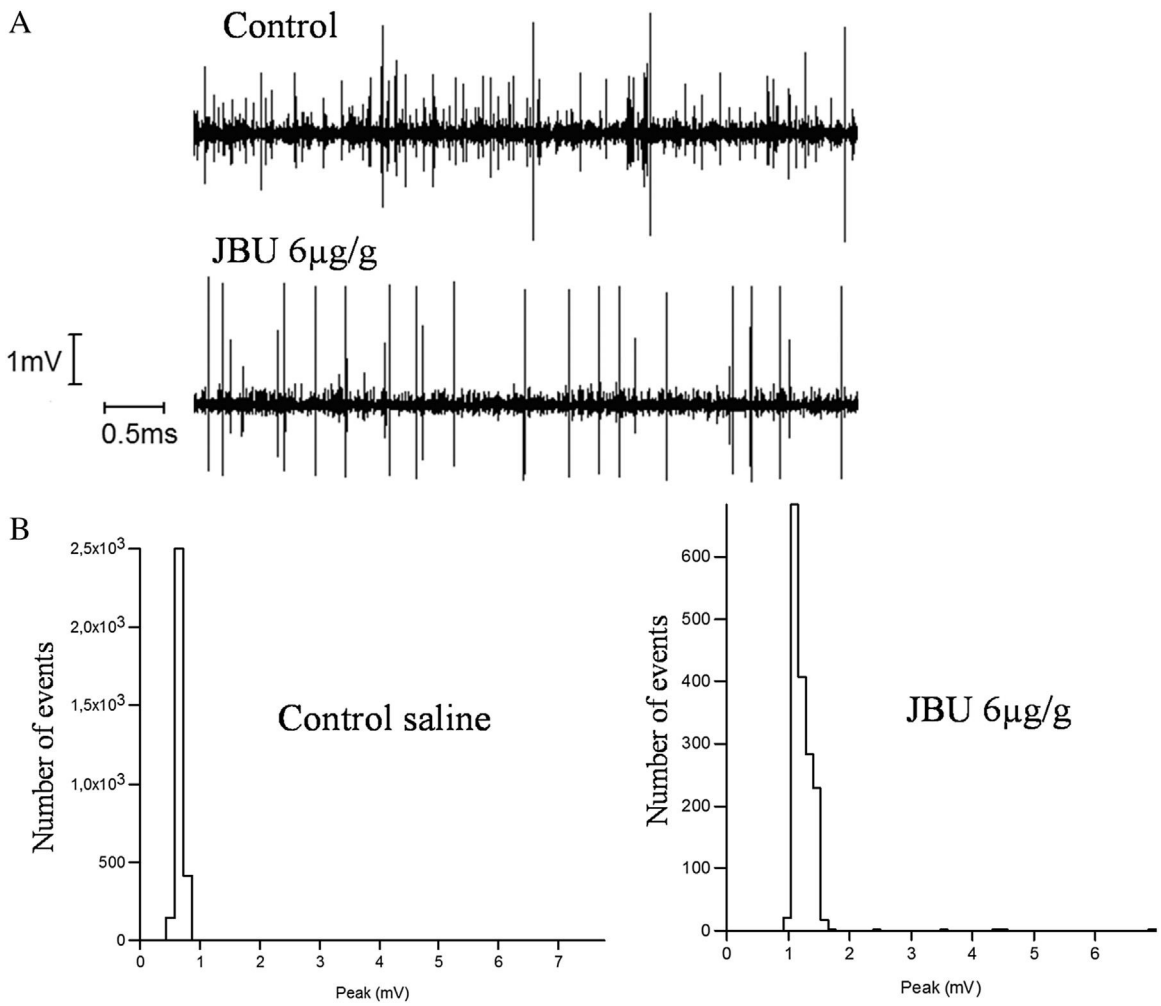

Fig. 7. Effect of Jack Bean Urease (JBU) on cockroach leg nerve-compound action potentials (SNCAP) kinetics.
Panel A shows representative traces of the SNCAP in control saline or JBU-treated preparations. Note the decrease in the frequency of the potentials during the 60 min recordings. In B, comparative histograms of control saline and JBU-treated preparations. Notice the increase in the frequency of higher amplitudes events upon exposure to JBU.

excitatory neurotransmitter and GABA as the main inhibitory one (Briley et al., 1982; Huber et al., 1990; Osborne, 1996). In insects the release of GLU or GABA in the synaptic cleft may induce either increase or decrease of muscle contraction strength, depending on the type of muscle and receptors present in the neuromuscular junctions (Chapman, 2013). Thus, the neuromuscular blockade produced by JBU in *N. cinerea* could result either from an increase of GABA-ergic neurotransmission or by inhibition of the glutamatergic counterparts. Considering that pretreatment of the insects with bicucculine decreased the level of neuromuscular blockade induced by JBU, it is suggested that GABA is involved in the inhibitory activity of the protein at the neuromuscular junction (Buckingham et al., 2005).

In our experimental model, the neuromuscular twitches were obtained by stimulating the nerve 5. In cockroaches, the axon of the slow depressor coxal motor neuron (Ds) leaves the ganglion via nerve 5 and innervates the coxal depressor muscle (muscle 177D) (Carr and Fourtner, 1980). Immunostaining studies with the locust *Schistocerca gregaria* revealed that at least two branches of inhibitory (GABA-ergic) neurons depart from nerve 5 at the metathoracic ganglion (Watson, 1986). Along nerve 5, the activity is conducted centrally (coming from afferent signals of sensila) or

peripherally, toward the methatoracic ganglion through monosynapticaly connected motorneurons via cholinergic synapses (Carr and Fourtner, 1980). Thus, we suggest that at least in part, the neuromuscular blockade induced by JBU in *N. cinerea* is consequent to an altered interplay between cholinergic motoneurons and GABA-ergic interneurons when in the presence of the protein.

JBU induced a decrease in the frequency of SNCAP concomitant with an increase in their amplitude. The hair plate neurons located on the animal's leg are connected to the ganglion via nerve 5, therefore the anti-AChE activity of JBU could also account for the increase in the amplitude of the spikes. Thus, it is possible that somehow JBU is inducing hyperpolarization of some branchs of nerve 5 either by increasing GABA-modulated activity or by direct interaction with neuronal membranes, resulting in an increased calcium-activated potassium conductance (French, 1986; Laurent and Hustert, 1988).

Finally, in some experiments we observed that higher doses of JBU produced less effect than lower doses. In fact, U-shape dose-response curves have been observed for JBU in other studies (Stanisçuaski et al., 2009). In Follmer and cols (Follmer et al., 2004), we reported that JBU undergoes a concentration-dependent oligomerization process which may lead to the formation of less active oligomeric states. Another possibility to explain the inverted dose-dependency of JBU effects could be that urease is acting as an inverted agonist. In such a condition, the effect attributed to the active form of a JBU's "receptor" with high affinity and efficacy would be counteracted by an increase of urease concentration and its consequent binding to an inactive form of the receptor displaying lower affinity and efficacy (Milligan, 2003).

5. Conclusions

Taken together our results indicate that Jack Bean Urease induces profound behavioral alterations in *Nauphoeta cinerea* cockroaches which may be related to the its anti-AChE-like activity and boosting of a secondary modulation of monoaminergic systems. The blockage of neuromuscular activity promoted by JBU is suggestive of an interplay between ACh and GABA signiling pathways in cockroaches. Further electrophysiological and biophysical studies on the direct interactions of Jack Bean Urease with the *Nauphoeta cinerea* nervous system are under way aiming to unveil more details of the molecular mechanisms involved in neurotoxic activity of this protein.

Conflict of interests

The authors declare no conflict of interests regarding this work.

Acknowledgments

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4. RESULTADOS – CAPÍTULO II

4.1. Manuscrito

Thiago Carrazoni, Christine Nguyen, Lucas F. Maciel, Andres Delgado-Cañedo, Brian A. Stewart, Angela B. Lange, Chariston A. Dal Belo, Celia R. Carlini, Ian Orchard.

"Jack bean urease modulates neurotransmitter release on insect neuromuscular junction."

Jack bean urease modulates neurotransmitter release on insect neuromuscular junction

Thiago Carrazoni[1,2,4], Christine Nguyen[2], Lucas F. Maciel[3], Andres Delgado-Cañedo[3], Brian A. Stewart[2], Angela B. Lange[2], Chariston A. Dal Belo[3], Celia R. Carlini[1,4], Ian Orchard[2].

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Introduction

During their evolution, plants have developed a vast range of mechanisms in order to defend themselves against the continuous threat posed by phytophagous insects. This defense armory ranges from morphological features to the production of entomotoxic compounds, such as toxic proteins and peptides (Vandenborre et al. 2011; Grossi-de-Sá et al. 2017). These natural compounds may affect a number of insect species by binding to different targets within the animal, including the nervous system. In insects, alterations in nervous system homeostasis can lead to neuroexcitation, resulting in hyperactivity, tremors, rigid paralysis and muscular fatigue (Rattan 2010). On the other hand, plant-derived compounds can also induce neuroinhibition that may result in paralysis and reduced respiratory capacity ultimately leading to the insect's death (Rattan 2010; Casida & Durkin 2013). Among plants defense-related compounds, ureases have been recently recognized as natural insecticides against some insect species (Carlini & Grossi-De-Sá 2002; Carlini and Polacco 2008; Carlini & Ligabue-Braun 2016). Ureases are metalloenzymes that catalyze the hydrolysis of urea into ammonia and carbon dioxide (Dixon et al. 1975). These enzymes are synthesized by bacteria, fungi and plants (Follmer 2008). In plants, the main role of ureases is related to the use of urea as nitrogen source (Eskew et al. 1984; Polacco et al. 2013). In this regard, the legume *Canavalia ensiformis* (jack bean) produces

different ureases isoforms, such as the major isoform jack bean urease (JBU or JBURE I) (Sumner 1926), canatoxin (CNTX) (Carlini & Guimaraes 1981; Follmer et al. 2001) and JBURE II (Pires-alves et al. 2003; Mulinari et al. 2011). Ureases display biological activities that are independent of their enzymatic activity (Follmer et al. 2001) such as platelet aggregation (Follmer et al. 2004; Olivera-Severo et al. 2006; Wassermann et al. 2010), antifungal activity (Postal et al. 2012) and entomotoxic effects (Defferrari et al. 2011; Stanisçuaski & Carlini 2012; Defferrari et al. 2014a; Carrazoni et al., 2016). The entomotoxic potential of JBU and derived peptides has been studied by our group for the past decade (Mulinari et al. 2007; Barros et al. 2009; Defferrari et al. 2011, Stanisçuaski & Carlini 2012; Defferrari et al. 2014a,b; Fruttero et al. 2016). Jack bean urease toxicity is variable, depending on the insect species, concentration and form of administration. Ureases were lethal when fed to insect species relying on cathepsin-like enzymes, as their main digestive enzymes, such as *Rhodnius prolixus* and *Callosobruchus maculatus*. On the other hand, they did not induce lethality in insects relying on trypsin-like enzymes such as *Schistocerca americana*, *Aedes aegypti*, *D. melanogaster* (Carlini et al. 1997; Stanisçuaski & Carlini 2012) or *Nauphoeta cinerea* (Carrazoni et al. 2016). Although peptides formed upon hydrolysis of plant ureases by insect cathepsin-like enzymes play an important role on their entomotoxicity (Carlini et al. 1997; Piovesan et al. 2008; Defferrari et al. 2011, Real-Guerra et al. 2013), it soon became clear that the whole protein is toxic itself, with no need of previous cleavage, interfering on insect's organs and systems (Stanisçuaski et al. 2009; 2010; Stanisçuaski & Carlini 2012; Carlini & Ligabue-Braun 2016).

One of the suggested mechanisms of JBU-induced toxicity is through the deregulation of calcium ion mobility across the cell membrane (Stanisçuaski et al. 2009, 2010; Martinelli et al. 2014; Carrazoni et al. 2016). The importance of calcium ions in JBU-induced toxicity was evaluated using the Malpighian tubule secretion and hemocyte aggregation assays. It was observed for both preparations that JBU toxicity was reduced when Ca^{2+} concentration was decreased (Stanisçuaski et al. 2009; Defferrari et al. 2014b). Moreover, although not cytolytic, JBU induces the formation of ion channels highly selective to cations in planar lipid bilayers and in lipid vesicles (Piovesan et al. 2014; Micheletto et al. 2016). The neurotoxic effects of JBU in cockroaches include neuromuscular blockage, whereas, upon *in vivo* administration, JBU leads to a progressive fade on muscle strength (Carrazoni et al. 2016).

There are multiple biological signals and structures regulating the neuromuscular junction in insects (Fulton & Usherwood 1977; Mellin et al. 1983; Elrick & Charlton 1999; Schwarz 2006; Rattan 2010; Desai & Lnenicka 2011; Casida and Durkin 2013). Depolarization caused by an action potential in the insect motor nerve terminal activates the calcium machinery through calcium channels and triggers an influx of calcium ions. These ions stimulate the release of a chemical transmitter, the aminoacid glutamate (Usherwood 1972; Cooper et al. 1996; Atwood et al. 1997). Free glutamate diffuses across the synaptic cleft and binds to a receptor-operated ion channel resulting in an influx of sodium and calcium ions (Clark et al. 1979; Desai & Lnenicka 2011). This influx subsequently activates the sarcoplasmic reticulum of skeletal muscle. The activated calcium channels then release an excess of calcium ions that lead to skeletal muscle contracture (Collet & Belzunces 2007). Based on our previous data regarding the interaction of ureases with insect physiological systems, we hypothesized the peripheral neurotransmission as a potential target for JBU-induced entomotoxicty. Here we aimed to gain a better understanding of the mechanism underlying the neuromuscular blockage induced by the *C. ensiformis* major urease (JBU) on the insect nerve-muscle communication.

Materials and methods

Chemicals

All chemicals and reagents were of the highest purity available and were obtained from Sigma-Aldrich, Merck, Roche, Life Technologies or BioRad. Test-solutions were prepared daily by dilution in locust saline (Orchard and Lange 1987), Drosophila saline (HL3) (Stewart 1994) or cockroach saline (Sturmer et al. 2014) immediately before use. Highly purified crystalline urease of *Canavalia ensiformis* (type C3) was obtained from Sigma-Aldrich Brazil. The protein (hexameric molecular mass 545 kDa) was dialysed against the insect solution used in each specific protocol. Stocks were kept at 4 °C and diluted in the appropriate insect saline before use.

Insects

Adult locusts (*Locusta migratoria*), cockroaches (*Nauphoeta cinerea)* and wandering third instar flies (*Drosophila melanogaster*) were employed as experimental models. *Drosophila* flies (Oregon-R wild-type strain) were kept at room temperature (23-25 ^{O}C) in a 12:12 h light/dark cycle and fed with Bloomington standard food medium. Locusts were raised under crowded conditions with a 12:12 h light/dark cycle at 30 °C and 50% humidity and fed with fresh wheat seedling and bran. Cockroaches were raised under crowded conditions, maintained at room temperature (23-25 °C) in a 12:12 h light/dark cycle with water and food (dog chow) *ad libitum*.

Lethality assay and weight gain

Second instar locusts (*L. migratoria*) were used to perform the lethality assay administrating JBU by feeding and injection. For the feeding protocol, animals were divided in control and JBU-treated groups and placed into containers (nine animals each, in triplicates). The animals were fed daily with lettuce disks (~30 mm diameter) one disks for each animal in the group, twice a day. A droplet of JBU was added to the surface of the lettuce disk for the JBU-treated group, in a final volume of 5 µL, and allowed to air dry before placing the disks inside the containers. Jack bean urease (~0.14 mg/animal/twice a day) diluted in locust saline (in mM) (NaCl 150, $CaCl_2$ 10, KCl 4, $MgCl_2$ 2, $NaHCO_3$ 4, HEPES 5, sucrose 90 and trehalose 5)(Orchard and Lange 1987) was used for the JBU-treated group. For the control group, only locust saline was added to the lettuce surface. Lethality rate and weight gain was measured at 24, 48, 72, 96, 120, 144 and 168 h after the beginning of the experiment. For the injection protocol, animals were also divided in control and JBU-treated groups and placed in containers, five animals each, in triplicates. Animals were injected in the abdomen near to the hind leg using a Hamilton syringe. For the JBU-treated group, JBU (0.28 mg/animal) diluted in locust saline was injected in a final volume of 5 µL. Control group was injected with locust saline only (Orchard and Lange 1987). Lethality rate was measured 12, 24, 48 and 72 h after the beginning of the experiment. Throughout the experiments animals were maintained in a 12:12 h light/dark cycle

at 30 °C and 50% humidity. For the injection protocol, the animals were also provided with food and water *ad libitum*.

Measurement of muscle potentials and nerve action potentials in Locusta migratoria

Muscle potentials from the tarsus depressor muscle of *L. migratoria* were recorded extracellularly from an isolated locust leg. The hind leg of an adult animal was removed and placed in a platform with attached electrodes. The stimulating electrodes were positioned in the femur and the recording electrodes were positioned in the tibia near the tarsus, with a ground electrode placed in between. Different treatments were administered through a window cut in the femur cuticle using a Hamilton syringe, in a final volume of 2.5 µL. Three doses of JBU were assayed: 0.0014, 0.014 and 0.14 mg/hind leg, and controls were injected with locust saline. Muscle potentials were recorded for 15 min prior to the injection of JBU. Each group consisted of 6 animals. Electrical stimuli were applied using a SD stimulator (model SD9B, Grass Technologies, Warwick, USA) at voltage of approx. 5 V, frequency of 0.2 PPS and a duration of 0.5 ms. The recordings were made using a differential amplifier (model 1700, A-M Systems, Sequim, WA, USA). Nerve action potentials (AP) of locust leg were measured in a similar preparation as for muscle potentials, using the same equipment. For the nerve AP, the position of stimulating and recording electrodes was inverted in this arrangement, since the stimulating electrode was placed in the tibia near the tarsus and the recording electrode was placed in the femur in order to acquire the action potential occurring in the tarsal fast depressor motorneurons (FDTa) (Hoyle and Burrows, 1973). Signal conversion was made using a PC-based data acquisition system (8 channels Powerlab, Dunedin, New Zeland), recorded with Chart software version 4 and the analysis made with Chart version 6.

Locust oviduct contraction assay

Oviducts from *L. migratoria* females were dissected along with the ovary and placed in a Sylgard-coated petri dish containing 400 µL of locust saline. The anterior part of the ovary was

pinned in the dish using minutien pins and the common oviduct located posteriorly was attached to a force transducer (model FT03, Grass Technologies, Warwick, USA) using a fine thread. The force of contraction of the lateral oviducts muscles, both left and right, was acquired using a PicoScope oscilloscope (model 2204, Pico Technology, Cambridgeshire, UK) and recorded using PicoScope software Picolog recorder. In this preparation, all the treatments were added to the bath solution in a volume of 200 µL, after the same volume of saline had been removed from the bath. Controls recordings were performed in the first 10 min, using locust saline only, followed by the addition of proctolin as a stimulating co-transmitter. Proctolin 10^{-9} M was applied for 5 min, then the preparation was washed with saline followed by the addition of 2 x 10^{-9} M proctolin plus 2 x 10^{-7} M JBU (final concentration 10^{-9} M proctolin and 10^{-7} M JBU). The preparation was washed again and proctolin alone was added followed by a final wash. All the wash out procedures were performed using locust saline.

The electrical evoked responses of the oviduct muscles were also evaluated, employing a similar protocol. Briefly, oviducts were dissected with the nerve that innervates the lateral oviducts (nerve N2B) still attached, the organ was placed in a Sylgard-coated petri dish, following the same steps as the protocol above, with the common oviduct attached to the force transducer by a fine thread. Afterwards the organ was placed in position, the end of the N2B nerve, either left or right, was placed into a suction electrode filled with locust saline and stimulated using 0.5 ms duration pulses by means of a World Precision Instruments stimulator (model A310, WPI, New Haven, CT, USA), inducing the lateral oviduct muscles to contract.

Miniature excitatory junctional potentials and excitatory junctional potentials in D. melanogaster larvae

The measurements of miniature endplate potentials (mEJPs) and excitatory junctional potentials (EJPs) were performed in third instar *D. melanogaster* wandering larvae neuromuscular junction as described by Nguyen and Stewart (2016). *D. melanogaster* larvae were dissected in HL3 solution (Stewart et al. 1994) with added $CaCl_2$. Glass intracellular electrodes were made using a micropipette puller (model P-97, Sutter instruments, Novato, CA, USA) and filled with 3 M KCl, the electrodes had a resistance of approximately 30 MΩ. For the mEJP protocol, the muscle number six from the body wall was impaled with the recording

electrode and the frequency and amplitude of the events were recorded. Only muscles with a resting membrane potential of -60 mV or below were used for the recordings. For the EJP protocol, the recordings were also made with muscle number six from the larvae body wall and the axons that innervate this muscle received an electrical stimulation of 1 mV/1 Hz, using a suction glass electrode, stimulus were delivered using an AxoClamp current and voltage clamp (model 2A, Axon Instruments, Sunnyvale, CA, USA). For both protocols the acquisition of the events was made using an AxoClamp amplifier (model 2B, Axon Instruments, Sunnyvale, CA, USA) and the analogic signal was converted to digital using an Axon Digidata (model 1322A, Axon Instruments, Sunnyvale, CA, USA). The recording of the events was carried out using pClamp 8.2 software and the analyses were performed using Clampfit 10 software. For the treated conditions, 10^{-7} M JBU was added in the bath solution along with different concentrations of $CaCl_2$. JBU was also assayed with calcium-free HL3 solution, containing the calcium chelating agent EGTA (ethylene glycol tetraacetic acid), and with $CoCl_2$ (Cobalt is a well-known calcium channel blocker). The preparations were pre-incubated with JBU for 1 min before the beginning of the recordings. Controls were recorded in the same preparations before the addition of JBU in the bath. For the mEJP protocol, the recordings had a duration of 2 min and for the EJP protocol the duration was calculated in sweeps (16 sweeps).

Calcium imaging

All imaging experiments were performed on a digital epifluorescence imaging system (WinFluor, J. Dempster, University of Strathclyde, Glasgow, Scotland) mounted on an Olympus IX71 microscope (Olympus America, Center Valley, PA, USA) using a 20X objective. Calcium imaging was performed using ventral nerve cords (VNC) from cockroaches (*N. cinerea*). After dissected out of the animals, the VNCs were loaded with Fluo-4 AM (5 µM, 45–60 min, at 32 ºC in an incubator) prior to the assays. Experiments were performed with VNCs continually perfused (1–2 mL.min-1) with cockroach saline solution with the following composition (in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, D-glucose10, pH7.4, 310mOsm, at room temperature. All compounds investigated were added via the perfusate. Data were calculated as changes in fluorescence ratio and expressed as $\Delta F/F_0$.

Statistical analysis

The results were expressed as mean ± S.E.M. Each experiment was repeated at least three times. For comparison between means of two different experimental groups the Student "t" test was employed. ANOVA was employed to analyze data from more than two experimental groups followed by Bonferroni multiple comparison as *post hoc* test. All statistical analyses were performed using GraphPad Prism6.0 (GraphPad Software, San Diego, CA, USA). The values were considered significantly different when $p \leq 0.05$.

Results

Lethality assay and weight gain

JBU was administrated to *L. migratoria* second instar locusts by injection and feeding. Jack bean urease at a daily dose of (0.28 mg/animal) was able to significantly reduce the animal weight gain, when administered orally. At the end of day 7 the animals displayed approximately 20% less weight when compared to controls (Figure 1). No lethality was observed neither when the animals were fed nor injected with JBU (0.28 mg/animal) (data not shown).

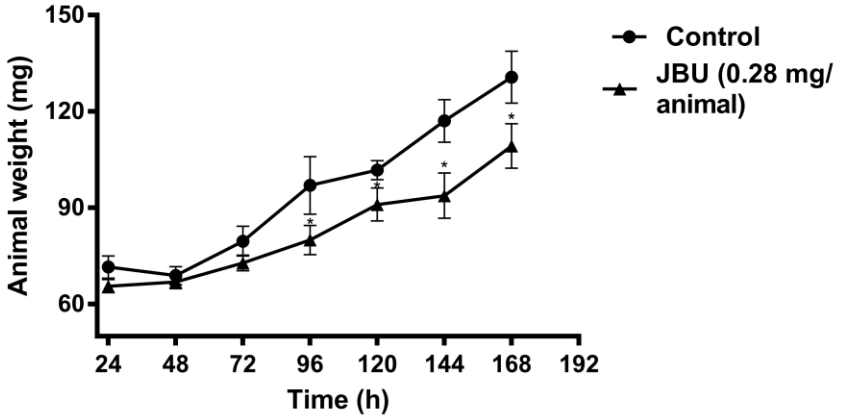

Figure 1. Effect of JBU feeding on *Locusta migratoria* weight gain. Second instar *L. migratoria* locust were fed with JBU (0.28 mg/animal) added daily to lettuce disks during seven days. The animal's weight were recorded each day before feeding. Graph of the mean ± S.E.M.* $p \leq 0.05$ Student "t" test, (n=9).

Muscle potentials and nerve action potentials

JBU induced a dose and time-dependent decrease in the amplitude of locust (*L. migratoria*) muscle potentials. The highest JBU dose assayed in this preparation (0.14 mg/hind leg) significantly reduced the amplitude of the muscle potential by 50% in approximately 30 min and by 70% after 60 min, compared to the control condition (Figure 2A). Control group was injected with locust saline solution. At the same dose in which JBU decreased the amplitude of muscle potentials no significant change in the amplitude of the conduction of nerve action potential was observed (Figure 2B).

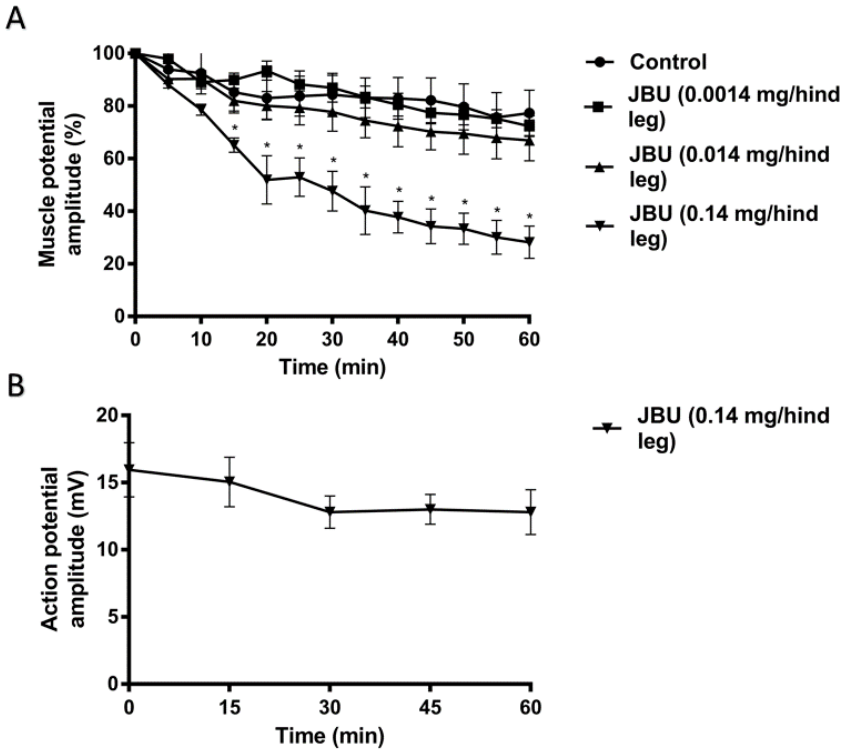

Figure 2. Effect of JBU on *L. migratoria* tarsal depressor muscle potential and leg nerve action potential. **(A)** The amplitude of the tarsus depressor muscle potentials was measured through extracellular recordings. Jack bean urease was assayed in three different doses: 0.0014, 0.014 and 0.14 mg, injected *ex vivo* in the locust hind leg in a final volume of 2.5 µL. Data are means ± S.E.M.* $p \leq 0.0001$ two-way ANOVA followed by Bonferroni's multiple comparison test (n=5). **(B)** The amplitude of the leg nerve action potential was measured using extracellular recordings. JBU (0.14 mg) was injected *ex vivo* in the locust hind leg in a final volume of 2.5 µL. The mean of first 15 min of each preparation was taken as control. $p \geq 0.05$ Student "t" test (n=6).

Locust oviduct contraction assay

Proctolin is a neuropeptide produced by different insect species and used as a co-transmitter in visceral and skeletal muscles, stimulating muscle contraction (Orchard, et al. 1989). Proctolin was applied in order to induce a strong muscle contraction without the need of electrical stimulation. JBU 10^{-7} M did not reduce the force of the muscle contraction induced by proctolin (Figure 3), neither the contraction induced by electrical stimulation (data not shown).

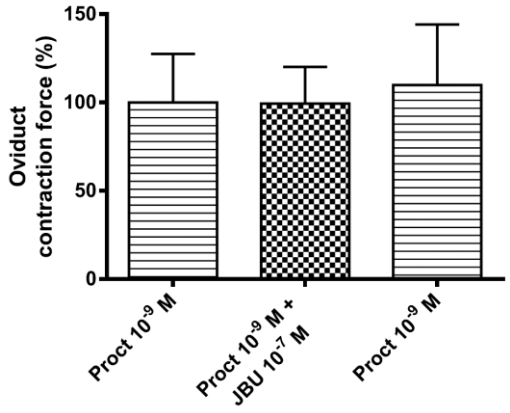

Figure 3. ***L. migratoria*** **oviduct contraction essay.** The strength of contraction of the oviduct muscles of a female *L. migratoria* was measured using a force transducer coupled to an oscilloscope. Muscle contractions were obtained using the peptide proctolin (10^{-9} M), in order to evoke consistent contractions. JBU was assayed at a concentration of 10^{-7} M. All treatments were added in the locust saline bath in the following order: Proctolin 10^{-9} M – wash – proctolin 10^{-9} M + JBU 10^{-7} M – wash – proctolin 10^{-9} M – wash. Data are means ± S.E.M. $p \geq 0.05$ Student "t" test (n=7).

Miniature endplate potentials and excitatory junctional potentials in D. melanogaster larvae

The effects of JBU were analysed in the neuromuscular junction of *D. melanogaster* larvae under different calcium conditions. JBU (10^{-7} M) in the presence of 1 mM $CaCl_2$ induced an increase in the frequency of mEJPs events in all amplitude intervals and also increased the magnitude of the events (Figure 4A). In this set of protocols it was also observed an overall increase in frequency of mEJPs (Figure 4B). The dependency of effects of JBU on external calcium was investigated. When the concentration of $CaCl_2$ in the medium was reduced to 0.4 mM, JBU (10^{-7} M) was unable to significantly increase the amplitude of mEJPs events (Figure 4C and 4D). The effect of JBU in the absence of external calcium was tested by employing a

HL3 solution with no added $CaCl_2$ and containing 10 mM EGTA, a calcium chelating agent (calcium-free condition). In contrast, JBU (10^{-7} M) reduced the frequency of events when added to a preparation in Ca^2-free conditions (Figure 4E and 4F). Although not statistically significant, a clear trend to a decrease in the number of events was observed when the preparation was treated with 10 mM $CoCl_2$, (Figure 4G and 4H). No EJPs were observed in the presence of 10 mM $CoCl_2$ (data no shown). On the other hand, JBU (10^{-7} M) induced an increase in the amplitude of EJPs events in the presence of 1 mM $CaCl_2$ when compared to control conditions for the same muscle (Figure 5A). The amplitude of the events was two-fold higher in the presence of JBU than in control conditions, as visualized Figure 5B. JBU (10^{-7} M) did not induce any significant changes in the amplitude of EJPs events in a low (0.4 mM) $CaCl_2$ medium (Figure 5C and 5D). No EJPs were observed in calcium-free condition.

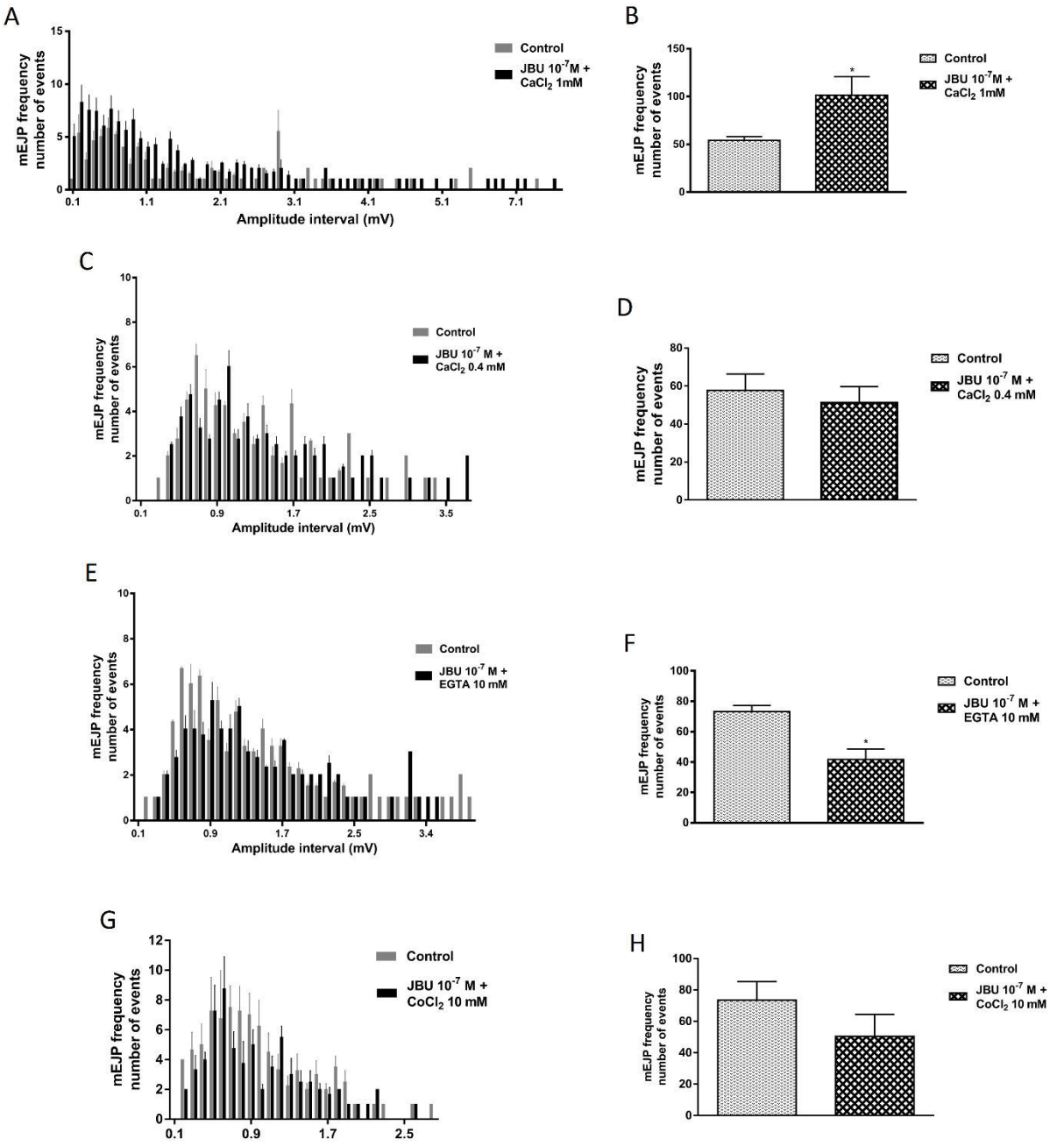

Figure 4. Effect of JBU on the frequency of miniature Excitatory Junctional Potentials (mEJPs) in *D. melanogaster* larvae neuromuscular junction. The frequency of spontaneous miniature Excitatory Junctional Potentials (mEJPs) was analysed by intracellular electrophysiological recordings of the 6th body wall muscle in *D. melanogaster* larvae. Panels **(A, B)** show an experiment conducted in the presence of 1 mM $CaCl_2$. **(C, D)** Experiment conducted in the presence of 0.4 mM of $CaCl_2$. **(E, F)** Experiment conducted in calcium-free condition. **(G, H)** Experiment conducted in calcium-free condition containing 10 mM $CoCl_2$. Panels **(A, C, E, G)** show the frequency by amplitude interval of mEJPs events. **(B, D, F, H)** Overall frequency of all mEJPs events, mean ± S.E.M. All experiments were performed at room temperature. * $p \geq 0.05$ Student "t" test (n=5).

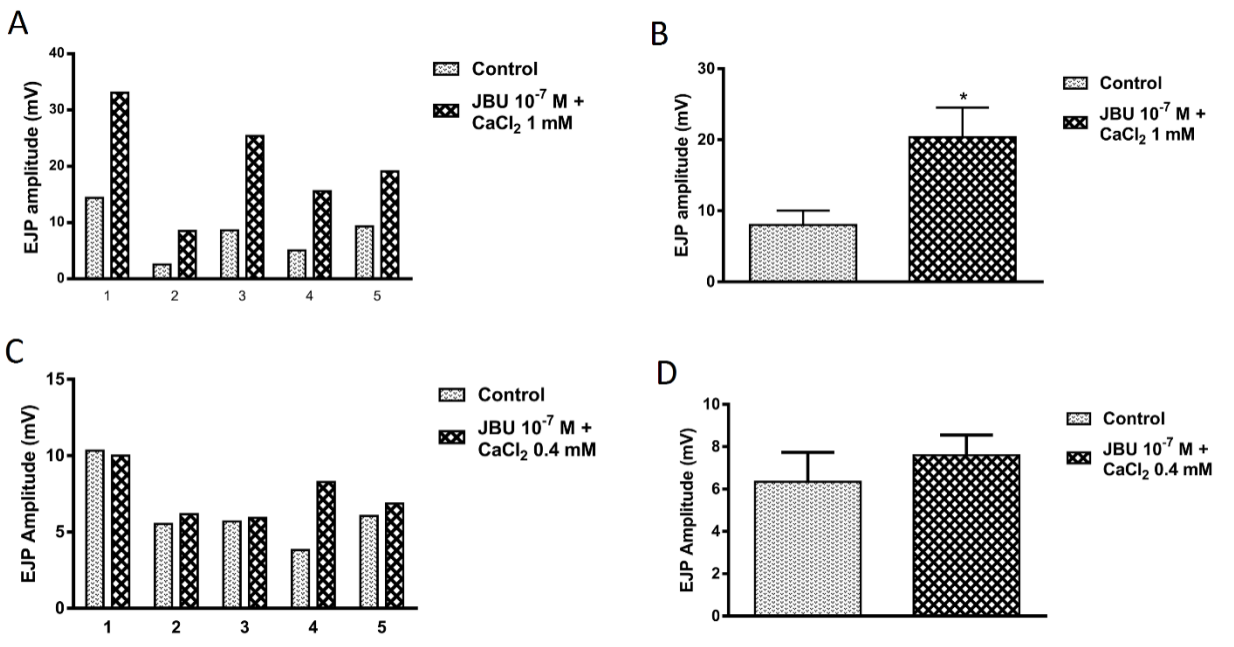

Figure 5. Effect of JBU on the amplitude of excitatory junctional potentials (EJPs) in *D. melanogaster* larvae neuromuscular junctions. The amplitude of EJPs was analysed by intracellular electrophysiological recordings of the 6th body wall muscle in *D. melanogaster* larvae. Panels **(A, B)** show experiments in the presence of 1 mM $CaCl_2$. **(C, D)** Experiment was conducted in the presence of 0.4 mM $CaCl_2$. Panel **(A, C)** show the amplitude of EJPs events during the onset of JBU activity compared to the control conditions in the same muscle (before and after), the x axis represents the number of preparations. On **(B, D)** overall amplitude of all EJPs events, mean ± S.E.M. All the experiments were performed at room temperature. * $p \geq 0.05$ Student "t" test, (n=5).

Increase of calcium influx

By using calcium imaging to monitor intracellular calcium levels ($[Ca^{2+}]i$), it was shown that JBU (1 µM) induced a pronounced rise in ganglionic $[Ca^{2+}]i$ (350 ± 50% peak increase (n=7), $p < 0.01$, Figure 06 B, D and E). Interestingly, depolarizing the neurons by applying high potassium (35 mM) after JBU infusion caused a further increase in the fluorescence revealing still more calcium uptake by the ganglia (Figure 06 C, D and E).

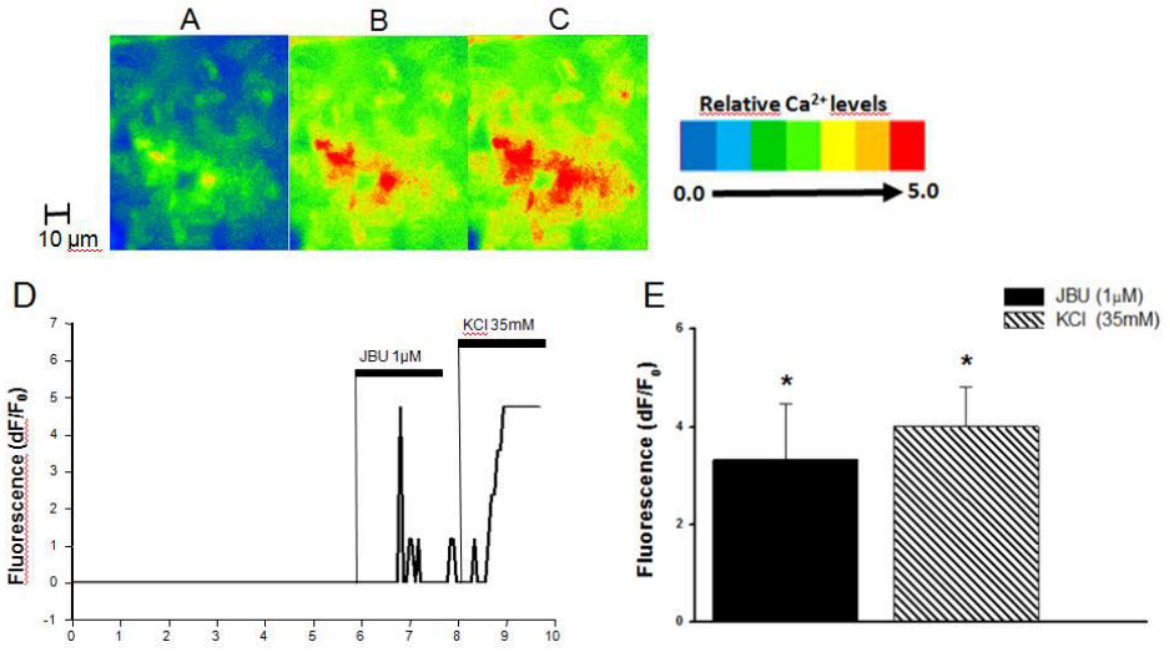

Figure 6. Increase of Ca^{2+} influx ($[Ca^{2+}]_i$) induced by Jack Bean Urease (JBU) in the third methatoracic ganglion of *Nauphoeta cinerea* cockroaches. Top panels show: **(A)** representative images of the methatoraxic ganglion loaded with Fluo-4/AM during baseline, **(B)** 2 min exposition to 1µM JBU and **(C)** 35 mM KCl perfusion, respectively. **(D)** is a representative graph showing the kinetic of fluorescence emission ($\Delta F/F_0$) induced by 1 µM JBU, followed by a subsequent addition of 35 mM KCl. (E), means ± S.E.M. of the maximum fluorescence emission during the perfusion of the ganglion with JBU or KCl (n=7). **p* <0.01 compared to baseline (n=7).

Discussion

This study aimed to improve our understanding regarding the mechanisms by which *C. ensiformis* urease (JBU) interferes on the insect nerve-muscle communication.

We started by investigating the survival rate and toxic effects on adult locusts by injecting and feeding the animals with JBU. No lethality was observed either by injection or feeding, however the animals showed a significant decrease in weight gain when fed with JBU. For *L. migratoria*, until now, there is not a good understanding about the nature of the neural components controlling feeding. According to Chapman and De Boer (1995) the overall level of feeding in *L. migratoria* is controlled by a neural network regulating feeding behaviour directly influenced by: (a) a short-term rhythm of running (15 min); (b) declining inhibition from the previous meal; (c) defecation; (d) central excitation, generated during feeding in proportion to the level of inhibition remaining from the last meal, and to chemical properties of the food; (e)

negative feedbacks (gut stretch, rapid osmotic and nutrient feedbacks) and finally (f) inhibitory inputs (continued gut stretch, hormonal effects, the influence of hemolymph osmolarity and nutrient titers). In addition, in *Locusta* the ganglia of the Stomatogastric Nervous System (STNS) (frontal ganglion, hypocerebral ganglion and ventricular ganglia) control movement of the foregut. Cutting the nerves from the frontal ganglion to the gut decreases feeding activity, suggesting its involvement in the animal feeding (Robertson et al. 2012). Thus, in our experimental conditions it is possible that a potential depolarizing activity induced by JBU over the nerves connecting frontal ganglion to the gut, could be involved in the reduced weight gain due to urease feeding in *L. migratoria*. The observed lack of lethal effect of JBU on locust was expected. In a previous work, we have reported that an isoform of JBU (canatoxin) was not lethal and did not it alter the weight gain when fed to another Orthoptera, *Schistocerca americana* (Carlini et al. 1997). *L. migratoria* also uses mainly trypsin-like enzymes as digestive enzymes (Sakal et al. 1989; Lam et al. 2000) which could account for the lack of lethality through the feeding pathway. On the other hand, there may be differences between the ureases isoforms as well as between digestives enzymes of the insects to justify the distinct effects on weight gain, which require further investigation.

In a previous work we reported the central and peripheral neurotoxicity induced by JBU in cockroaches *N. cinerea*, leading to a dose and time-dependent neuromuscular blockage (Carrazoni et al. 2016). Based on that data, we hypothesized that JBU's neurotoxicity could involve alterations of the release of neurotransmitters at the insect neuromuscular junction. In order to confirm this hypothesis, here we wanted to unveil if the main locus of JBU activity is postsynaptic (on the muscle) or presynaptic (at the level of the peripheral nerves).

The effects of JBU on nerve-muscle communication were assayed using extracellular electrophysiological recordings in the tarsal muscle of *L. migratoria* and intracellular electrophysiological recordings of the 6th body wall muscle of *D. melanogaster* larvae. In the locust preparation the action potentials generated through electrical stimulation are conducted along two motor neurons which innervate the tarsus depressor muscle (Personius & Chapman 2002). When injected into the locust's leg, JBU produced a dose and time-dependent decrease on the amplitude of the muscle potentials, which goes along with the neuromuscular depression observed *in vivo* on *N. cinerea* muscle-nerve preparations (Carrazoni et al. 2016). However,

when JBU was assayed directly on isolated nerve and isolated muscle preparations, no significant decrease of the amplitude of leg nerve APs was seen nor of the twitches of the lateral oviduct muscles, as evoked by proctolin or electrical stimulation. The hindleg of a locust is innervated by the thoracic nerves (3N3, 3N4, 3N5), originating in the methathoracic ganglion (Bharadwaj & Banerjee 2010). Extracellular recordings from these nerves showed no alteration in the APs conductance under the influence of JBU. The absence of inhibition of the nerve AP conductance suggests that the insect leg muscle is the main site for the JBU inhibitory activity. Locust oviduct muscles are myogenic muscles under the influence of neuromodulators such as octopamine, proctolin and glutamate, being the latter a classical excitatory neurotransmitter in invertebrates (Orchard & Lange 1986). In our experimental conditions, JBU was added to the bath of an *in vitro* oviduct muscle preparation in which contractions were evoked both by proctolin (Orchard et al. 1989) or electrical stimulation on the oviduct motor neuron (Orchard & Lange 1987). In this set of protocols, unlike the assay with the tarsal muscle, JBU was ineffective to reduce the amplitude of muscle twitches. Accordingly, in both preparations the muscles receive inputs from motor neurons, being the tarsus muscles modulated mainly by glutamate (excitatory) and γ-aminobutyric acid (GABA) (inhibitory) (Osborne 1996). On the other hand, besides receiving glutamatergic inputs the oviductal muscles also respond to other neuromudulators, such as proctolin and octopamine (Orchard & Lange 1987). Thus, the latter result reinforces the involvement of GABA and GLU in the depressive activity of JBU on insect neuromuscular junctions.

In order to better understand the effect of neurotoxicity of JBU we performed electrophysiological recordings in *D. melanogaster* neuromuscular junction. JBU added to the bath preparation induced an increase in the frequency of spontaneous mEJPs and in the amplitude of EJPs in the presence of 1 mM of $CaCl_2$. When the $CaCl_2$ concentration in the bath was lowered to 0.4 mM, JBU was unable to alter either mEJP or EJP events. When using calcium-free HL3 along with a calcium chelator, the effects of JBU were abolished indicating that the calcium mobility in the motor neuron plays a central role in JBU-induced effect. In the neurotransmission, the increase in the frequency and in the amplitude of the synaptic events indicates that more vesicles of neurotransmitter are being released from the pre-synaptic terminal (Schwarz 2006). An excess of neurotransmitter release, as frequently observed during the activity of certain neurotoxins, such as snakes toxins (Dal Belo et al. 2005) and scorpions toxins

(Quintero-Hernandez et al. 2008; Stevens et al. 2011), can cause hyperexcitation that might lead to neurotransmitter run out (Casida & Durkin 2013), similar to the effect of spider toxins on the neuromuscular junction of crayfish and insect (Dulubova et al. 1996; Umbach et al. 1998; Holz & Habener 1998; Elrick & Charlton 1999) or in a prolonged depolarization of the post-synaptic terminal (Cull-Candy 1976; Dal Belo et al. 2005). This phenomenon ultimately results in neuromuscular failure, which could be also an explanation for the neuromuscular blockage produced by JBU activity (Carrazoni et al. 2016). Such physiological event is commonly preceded by an increase in the influx of Ca^{++} in nerve terminals. Indeed, in our experimental conditions JBU induced an increased influx of Ca^{2+} in the cockroach methatoracic ganglion. This observation gives support to the hypothesis that JBU activity leads to exhaustion of the pre-synaptic terminal which further develops into neuromuscular failure. In this context, it is worth to mention other biological effects of ureases (including JBU isoforms) for which interference on calcium mobility across membranes had been implicated, such as platelet aggregation and induction of exocytosis (Olivera-Severo et al. 2006; Wassermann et al. 2010; Carlini & Ligabue-Braun 2016), and antifungal properties (Postal et al. 2012). The antidiuretic effect of JBU in *Rhodnius prolixus* was studied using a Malpighian tubule secretion assay, and revealed disruption of calcium flux across membranes as part of the inhibitory mechanism (Stanisçuaski et al. 2009)

Finally, the ability of JBU insert itself into lipid membranes (Micheletto et al. 2016) and to form cation-selective ion channels in planar lipid bilayers (Piovesan et al. 2014) also support a depolarizing activity that might increase quantal release over nerve terminals, similarly to that produced by the pore-forming toxin pardaxin of the flatfish *Pardachirus marmoratus* (Renner et al. 1987). Whether the neuromuscular blocking activity of JBU is through a depolarizing activity over the pre-synaptic terminals involving the activation of voltage-gated Ca^{2+} channels or directly through a pore-forming activity still remains to be answered.

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5. DISCUSSÃO GERAL E CONCLUSÃO

Nessa tese os estudos da atividade entomotóxica da enzima JBU geraram novos conhecimentos acerca do mecanismo de ação da toxina sobre o sistema nervoso de insetos, além de trazer novas evidências que corroboram hipóteses propostas anteriormente.

Após o tratamento de baratas da espécie *N. cinerea* com a urease majoritária da leguminosa *C. ensiformis* (JBU) observou-se que, apesar de não ser letal para o animal, a proteína induziu uma redução na atividade locomotora, quando comparado com animais controle. Além disso, quando a urease foi administrada via oral para gafanhotos da espécie *L. migratoria* os mesmos demonstraram uma redução no ganho de peso. Segundo Chapman & De Boer (1995), o ganho de peso em insetos é controlado por uma rede neural influenciada diretamente pela atividade locomotora, estímulos externos (ex.: disponibilidade de alimento) e atividade hormonal (ex.: saciedade). Além do sistema SNC, o sistema nervoso estomatogástrico (que controla as contrações do intestino do animal) também pode influenciar na alimentação do mesmo. De acordo com Robertson e colaboradores (2012), ao seccionar os nervos que conectam o gânglio frontal ao intestino há uma inibição na alimentação, sugerindo o envolvimento desses gânglios no comportamento de alimentação do animal. Portanto, um possível efeito da JBU sobre esses gânglios ou nervos poderia induzir a redução de locomoção vista em *N. cinerea* e também a alteração observada no ganho de peso de *L. migratoria*.

Em uma análise a nível bioquímico dos efeitos da urease no sistema nervoso de *N. cinerea*, foi observado que a administração da JBU induziu uma inibição da enzima acetilcolinesterase (AChE) em gânglios cerebrais. A AChE, assim como em vertebrados, é a enzima responsável pela inativação do neurotransmissor acetilcolina (ACh), que também atua como neurotransmissor no SNC (Osborne 1996). A inibição da enzima AChE produz uma hiper-estimulação da neurotransmissão colinérgica do animal, que acaba por alterar a liberação de outros neurotransmissores, tais como octopamina (OA) (Libersat 2010). O neurotransmissor OA está presente em diferentes tecidos do inseto (Axelrod & Saavedra 1977), sendo que as maiores concentrações estão presentes no SNC (Evans 1978), onde pode atuar como neurotransmissor, neurohormônio e neuromodulador (Orchard 1982). Um dos papeis atribuídos a OA é a

modulação de comportamentos do inseto (Verlinden et al. 2010), tais como o comportamento de *grooming* (ato de limpeza dos órgãos sensoriais, ex.: antenas e pernas, utilizando o aparelho bucal) (Weisel-Eichler et al. 1999). Acredita-se ainda que OA também esteja envolvida na transmissão pré-motora no gânglio metatoráxico do inseto (Libersat et al. 2009). Nesse sentido, em nossas condições experimentais, baratas *N. cinerea* tratadas com a JBU demonstraram um aumento no tempo que o animal gasta realizando o comportamento de *grooming*, em paralelo ao desenvolvimento de um bloqueio neuromuscular parcial. Dessa forma, a influência da JBU sobre a transmissão colinérgica poderia também alterar a transmissão octopaminérgica e, indiretamente, alterar a resposta motora dos animais, por mecanismos ainda não confirmados, mas que poderiam envolver a neurotransmissão glutamatérgica e GABAérgica.

Além dos efeitos a nível central, a JBU também demonstrou atividade em neurônios aferentes (sensoriais) e neurônios eferentes (motores). O tratamento de preparações de neurônios sensoriais de *N. cinerea* com a JBU alterou a frequência e a amplitude dos potenciais de ação espontâneos nesses neurônios, indicando que a sua ação não está restrita apenas ao nível do SNC do inseto. A urease também induziu alterações dos potenciais musculares caracterizada pela diminuição da amplitude dos potenciais musculares evocados, tanto em *N. cinerea* quanto em *L. migratoria*. Por outro lado, não houve alteração na resposta contrátil do músculo do oviduto isolado de *L. migratoria*, assim como não houve alteração na condução do potencial de ação em nervos isolados do mesmo animal. Nesse contexto, nossos resultados sugerem que o efeito da JBU no SNP poderia estar ocorrendo a nível da junção neuromuscular.

Afim de analisar a hipótese de um efeito da JBU na junção neuromuscular, foram realizados ensaios eletrofisiológicos em larvas de moscas *D. melanogaster.* Nesses protocolos verificou-se que a aplicação da JBU induziu um aumento na frequência dos mEJPs e um aumento na amplitude dos EJPs, o que caracteriza uma atividade altamente dependente da concentração de íons de cálcio no meio extracelular (Karunanithi et al. 1997; Schwarz 2006; He et al. 2009; Mikov et al. 2015) Como dito anteriormente, a junção neuromuscular de insetos utiliza dois neurotransmissores principais, GABA (inibitório) e Glu (excitatório) (Osborne 1996). Assim, uma hiper-excitação do músculo por aumento na liberação de Glu induziria uma despolarização da membrana muscular, entretanto quando em excesso, Glu pode induzir uma resposta bifásica no músculo, uma rápida despolarização seguida de uma hiper-polarização

prolongada (Cull-Candy 1976), evento este que poderia acarretar na dessensibilização do terminal pós-sináptico (Clark et al. 1979; Dumbar & Piek, 1983).

O Glu é sintetizado e liberado a partir do terminal pré-sináptico do neurônio motor e atua sobre receptores específicos no músculo (Usherwood & Grundfest 1965; Usherwood 1967). A liberação de neurotransmissores na junção neuromuscular de insetos se dá de modo similar à liberação em vertebrados: os neurotransmissores são liberados a partir de vesículas que se fundem à membrana do terminal pré-sináptico, um processo chamando de *docking* ou ancoragem de vesículas, que é altamente dependente do influxo de cálcio para o terminal do neurônio motor (Elrick & Charlton 1999; Schwarz 2006; Milkov et al. 2015). De fato, em nossas condições experimentais, ensaios de imagem de cálcio em gânglio de *N. cinerea* demonstraram que a JBU induz um aumento no influxo de Ca^{2+} no neurônio, o que está de acordo com a hipótese de uma maior quantidade de neurotransmissores liberada pelo neurônio (Elrick & Charlton 1999).

6. PROPOSTA DE MECANISMO DE AÇÃO DA JBU SOBRE O SISTEMA NERVOSO DE INSETOS

Há três principais hipóteses que poderiam explicar como a JBU aumentaria o influxo de ions de Ca^{2+} para o terminal pré-sináptico. A primeira é que a JBU poderia atuar sobre um alvo em comum a todos os sistemas de neurotransmissão afetados, sendo que canais de Ca^{2+} voltagem dependentes da subfamília 2 (Ca_v2) poderiam ser este alvo, uma vez que estes canais estão localizados predominantemente no terminal do neurônio, e participam ativamente no processo de liberação de neurotransmissores (Catterall, 2011). Dessa forma, JBU poderia ligar-se diretamente ou pelo menos parcialmente ao Ca_v2, modulando o mesmo. A segunda hipótese é que ocorra uma inserção da própria JBU na membrana do neurônio levando à formação de poros na mesma. De fato, em estudos anteriores foi demonstrado que a JBU é capaz de inserir-se em membranas bilipídicas e formar canais iônicos seletivos a cátions (Piovesan et al. 2014; Micheletto et al. 2016). A terceira hipótese é que a JBU se insira na membrana sem induzir a formação de canais, entretanto esta inserção desestabilizaria a bicamada lipídica de modo a modular o funcionamento dos Ca_v2 presentes na membrana. Ainda que o exato mecanismo pelo qual JBU induz o aumento no influxo de Ca^{2+} permanece por ser esclarecido, quaisquer dos mecanismos supracitados poderia desencadear uma hiper-estimulação do terminal pre-sináptico, levando a um processo de esgotamento do neurotransmissor neste terminal, devido à falta de tempo hábil para a reciclagem de vesículas (Wildemann & Bicker 1999; Schwarz 2006; Rizzoli 2014) ou recaptação do neurotransmissor (Gardiner et al. 2002). O esgotamento do terminal pré-sináptico em si já seria capaz de induzir um bloqueio neuromuscular (Elrick & Charlton 1999). Ademais, o aumento na liberação do neurotransmissor também induziria uma persistente despolarização do terminal pós-sináptico ocasionando um eventual bloqueio neuromuscular por hiper-excitação, similar ao efeito induzido por certas toxinas animais (Dal Belo et al. 2005). A Figura 5 resume as hipóteses propostas nessa tese para explicar a ação neurotóxica da JBU em insetos, a partir dos resultados obtidos em *N. cinerea, L. migratoria e D. melanogaster.*

Dessa forma, concluímos que o efeito de bloqueio neuromuscular induzido pela JBU em insetos ocorre diretamente sobre os terminais pré-sinápticos de neurônios, tanto do sistema nervoso central quanto de neurônios aferentes e neurônios motores da junção neuromuscular.

Conclui-se ainda que a JBU induz um aumento do influxo de Ca^{2+} para o terminal do neurônio, liberando por consequência uma maior quantidade de neurotransmissor, o que poderia em última análise, induzir o esgotamento do terminal pré-sináptico e/ou uma persistente despolarização do terminal pós-sináptico, levando a um eventual bloqueio neuromuscular, um efeito similar aos já descritos para algumas toxinas animais (Renner et al. 1987; Kiyatkin et al. 1993; Dulubova et al. 1996; Holz & Habener 1998; Umbach et al. 1998; Elrick & Charlton 1999).

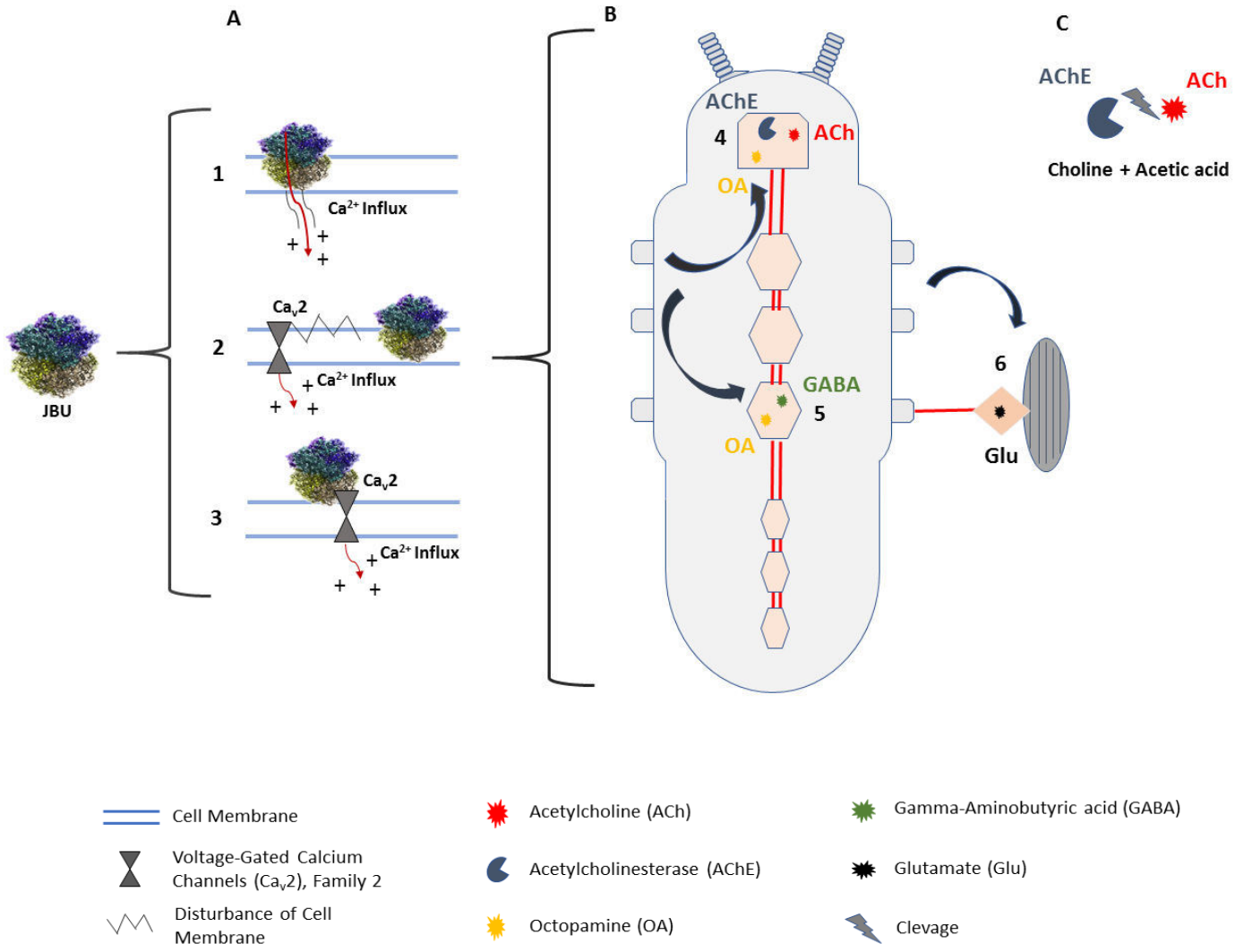

Figura 5. Proposta de mecanismo de ação da JBU sobre o sistema nervoso de insetos. (Painel A) sugestão de três possíveis interações da JBU com alvos na membrana de neurônios. **(1)** A urease poderia inserir-se na membrana do neurônio e formar canais seletivos a cátions; **(2)** A urease poderia inserir-se na membrana do neurônio próximo aos canais de cálcio voltagem dependentes da família 2 (Ca_v2), desestabilizando a camada bilipídica e modulando o funcionamento dos mesmos. A abertura dos Ca_v2 induz o influxo de Ca^{2+} para o interior do neurônio e a consequente liberação de neurotransmissores; **(3)** A urease poderia ainda ligar-se total ou parcialmente aos canais Ca_v2 modulando diretamente o funcionamento destes canais. Qualquer um dos mecanismos supracitados poderia induzir um aumento no influxo de ions Ca^{2+} para o interior do neurônio, com o consequente aumento na liberação de neurotransmissores. **(Painel B)** Sugestão de possíveis alvos da JBU no sistema nervoso central (SNC) do inseto. **(4)** A JBU poderia atuar diretamente nos gânglios do SNC, sobre a transmissão colinérgica, possivelmente através da redução a atividade da enzima acetilcolinesterase. **(5)** A modulação da transmissão colinérgica poderia modular outros sistemas de neurotransmissores que intercomunicam com o sistema colinérgico como, por exemplo, os sistemas octopaminérgico e GABAérgico. **(6)** Além de atuar no SNC, a urease também pode atuar diretamente no sistema nervoso periférico, mais especificamente sobre a neurotransmissão glutamatérgica na junção neuromuscular. A atuação da JBU, tanto no SNC quanto na junção neuromuscular, poderia induzir os efeitos observados como alterações comportamentais, alterações no ganho de peso, modulação dos eventos sinápticos e, por fim, bloqueio neuromuscular. **(Painel C)** A reação de clivagem da acetilcolina pela enzima acetilcolinesterase é necessária para que o neurotransmissor seja reciclado. No caso da inibição da atividade da enzima AchE observada em no tratamento com JBU, tal efeito poderia desencadear um processo de hiper-excitação da transmissão colinérgica do animal, alterando, por conseguinte, outros sistemas de neurotransmissão, o que acarretaria em uma neurotoxicidade generalizada.

7. REFERÊNCIAS BIBLIOGRÁFICAS

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Anexos

I. Artigo Publicado

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Participação na execução e análise de experimentos eletrofisiológicos nos quais foi mensurado o potencial de ação de neurônios aferentes de baratas sob influência do veneno de escorpião da espécie *Bothriurus bonariensis*.

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Bothriurus bonariensis scorpion venom activates voltage-dependent sodium channels in insect and mammalian nervous systems

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ABSTRACT

Animal venoms have been widely recognized as a major source of biologically active molecules. *Bothriurus bonariensis*, popularly known as black scorpion, is the arthropod responsible for the highest number of accidents involving scorpion sting in Southern Brazil. Here we reported the first attempt to investigate the neurobiology of *B. bonariensis* venom (BBV) in the insect and mammalian nervous system. BBV (32 μg/g) induced a slow neuromuscular blockade in the *in vivo* cockroach nerve-muscle preparations (70 ± 4%, n = 6, $p < 0.001$), provoking repetitive twitches and significantly decreasing the frequency of spontaneous leg action potentials (SNCAPs) from 82 ± 3 min^{-1} to 36 ± 1.3 min^{-1} (n = 6, $p < 0.05$), without affecting the amplitude. When tested in primary cultures of rat hippocampal cells, BBV induced a massive increase of Ca^{2+} influx (250 ± 1% peak increase, n = 3, $p < 0.0001$). The disturbance of calcium homeostasis induced by BBV on the mammalian central nervous system was not accompanied by cellular death and was prevented by the co-treatment of the hippocampal cells with tetrodotoxin, a selective sodium channel blocker. The results suggest that the biological activity of BBV is mostly related to a modulation of sodium channels function. Our biological activity survey suggests that BBV may have a promising insecticidal and therapeutic potential.

1. Introduction

Scorpions are broadly distributed among all continents, except the poles [1]. Differences in the venoms composition and in their injection apparatus represent evolutionary attributes that contributed to the adaptation of these animals to the many different terrestrial environments [2]. Although the primary purpose of arthropod venoms is to paralyze their natural prey, the insects, scorpions are one of the animals most frequently implicated in human envenomation throughout America, Asia, and Africa [3]. However, the sting of only about 25 out of almost 1500 species of scorpions results in severe envenomation to humans.

Abbreviations: BBV, *Bothirurus bonariensis* venom; 4-AP, 4-aminopyridine; TTX, tetrodotoxin citrate; DIV, days *in vitro*; HBS, HEPES-buffered saline; GLU, glutamate; SNCAP, spontaneous neural compound action potentials; NCX, Na^+/Ca^{2+} exchangers; CaVs, voltage-gated Ca^{2+} channels; IP3, inositol triphosphate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNS, central nervous system; PNS, peripheral nervous system; VGSC, voltage-gated sodium channels.

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Every year, more than one million individuals are stung by scorpions worldwide [4]. In scorpionism, the clinical signs of the envenomation generally depend upon the species of scorpions involved, together with the amount of venom injected. Thus, local reactions are frequently associated to stings by nonlethal scorpions, while systemic symptoms are related to accidents provoked by lethal species. In Latin America the majority of deadly scorpions belong to the family Buthidae, which comprehends the *Tityus* genus. The envenomation by non-Buthidae scorpions (e.g *Bothriurus sp*.) in general does not produce serious symptoms [5]. Usually, their stings cause only inflammatory or immunological reactions in the victims [3]. However, in very few cases, the pain at the local of sting can progress to erythema, oedema, arterial hypotension and vomiting [6]. The severity of envenoming is related to age, and size of the scorpion, the season of the year, and to the time elapsed between sting and hospitalization [4].

Scorpion venoms are complex chemical mixtures containing a variety of toxic protein and non-protein toxins, enzymes, nucleotides, lipids, biogenic amines, and other unknown substances presenting biological activity [5]. Following a scorpion sting, envenomation normally causes neuronal excitation, mostly due to biding of certain scorpion α-toxins rather than β-neurotoxins to voltage-gated sodium channels [6]. Indeed, several neurotoxic peptides have been isolated and characterized from the venom of different species of scorpions [7], and their activity is often attributed to the modulation of ion channels [8]. An example of this type of chemical compound is phaiodotoxin, a toxin from the venom of the non-Buthidae scorpion *Anuroctonus phaiodactylus* shown to activate voltage-dependent sodium channels in insects [9]. Besides their obvious clinical interest, the specificity of certain scorpion toxins for binding to excitable tissues (e.g. nerves and muscles), makes them a rich source of compounds with biotechnological interest [10].

The scorpion *Bothirurus bonariensis* (C.L. Koch, 1842) (Fig. 1), popularly known as “black scorpion”, is commonly found in Southern Brazil [11]. Despite the clinical symptoms of *B. bonariensis* envenomation are generally not severe, this scorpion causes about 400–500 accidents every year [12]. Thus, here we investigated the activity of BBV on neurophysiological parameters on insect nerve preparations and mammalian hippocampal cell cultures.

Fig. 1. Representative picture of a *Bothriurus bonariensis* scorpion from São Gabriel, Rio Grande do Sul, Brazil.

2. Materials and methods

2.1. Reagents

All drugs were made up to at least 1000× stock and using appropriate dilutions were added via the perfusate or inject in *in vivo* experiments. Neurobasal-A Medium was purchased from (Invitrogen, UK). B-27 and Fluo 4/AM were from Thermo Fisher Scientific (São Paulo, Brazil). Poly-L-lysine, L-glutamine, cytosine-β-D-arabinofuranoside, and 4-aminopyridine (4-AP) were obtained from Sigma Aldrich Brazil (São Paulo, Brazil). Tetrodoxin citrate (TTX) was purchase from Alomone Labs (Jerusalem, Israel). All other chemicals and reagents employed in the experiments were of the highest purity and were obtained from Sigma, Aldrich, Merck, Invitrogen, and BioRad.

2.2. Scorpion collection and venom extraction

B. bonariensis specimens of both sexes were collected at the Campus of Federal University of Pampa, city of São Gabriel, Rio Grande do Sul, Brazil. The procedures were previously authorized by the Head of the Campus Dr. Valdir Marcos Stefenon, with the permission of the System of Authorization and Information in Biodiversity-SISBIO number SISBio nº 24867-2. The venom was obtained by an electrical stimulation of the scorpions with an electrical pulse (35 V, 1 ms, 10 Hz). After collection, the venom was frozen at −85 °C, lyophilized, and stored at the same temperature until use.

2.3. Cockroaches and newborn rats

All animal care and experimental procedures were in accordance with the guidelines of the National Council to Animal Experimentation-CONCEA. Adult male *Nauphoeta cinerea* cockroaches (3–4 month after adult molt) were reared at laboratory conditions of controlled temperature (22–25 °C) on a 12 h: 12 h L: D cycle. To estimate a precise dose/g of the different compounds assayed *in vivo*, two hundred animals were previously weighted, given a final body weight of 0.5 ± 0.03 g. Two-day-old Wistar rats of both sexes were used for the primary cultures of hippocampal cells. Pregnant female rats were purchased from the Centre of Biological Experimental Models from the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). The animals were kept under an alternate 12 h cycle of light/dark, at a constant 24 °C temperature, with access to water and food *ad libitum*. The newborn rats were kept with the dams until the moment of the sacrifice. A total of 12 newborn rats were used to conduct the cell cultures. Briefly, 1- to 2-day-old Wistar rats were killed by cervical dislocation and decapitated. The experiments were conducted at the Neurosciences Laboratory of the Brain Institute, at PUCRS. The study was performed according to and approved by the Ethics and Research Committee and Ethics Commission in Animal Use from PUCRS, under the protocol number 13/00340. No more than one offspring from each litter and dam were used in these experiments.

2.4. Primary cultures of rat hippocampal cells

Primary cultures of hippocampal cells were prepared as described previously [13]. Briefly, 1- to 2-day-old Wistar rats were killed by cervical dislocation and decapitated. The hippocampi were removed and triturated, and the resulting cells were plated at a density of 3×10^5 cells mL^{-1} onto 13 mm poly-L-lysine coated coverslips. Cultures were incubated in culture media consisting of Neurobasal-A Medium (Invitrogen, UK) supplemented with 2% (v/v) B-27 (Invitrogen, UK) and 2 mM L-glutamine and maintained in a

humidified atmosphere at 37 °C/5% CO_2 for 13–16 days *in vitro* (DIV). After 5 DIV, cytosine-D-arabinofuranoside (10 mM) was added to inhibit glial cell proliferation. Ca^{2+} imaging experiments were performed on cells taken from at least three separate cultures obtained from different rats.

2.5. Assessment of cell viability

To assess the cell viability, the colloidal dye (Tripan blue) exclusion method [14] was employed. This assay is based on the simple principle that viable cells will exclude the Trypan Blue dye, whereas dead or dying neurons will not, thus, appearing blue. Primary cultures of rat hippocampal cells of 10 DIV were incubated with the drug for 5 min, then exposed to the 0.2% Trypan Blue for 5 min and immediately counted *in situ* using bright field optics with a grid-containing eyepiece. We compared the viability of cells treated with BBV (4 μg/ml) with cells treated with HEPES-buffered saline (control of normal cell viability), and a control for cellular death, consisted of cells treated with 0.01% hypochlorite. The results were expressed as the percentage of cell dead after incubation with treatments.

2.6. Ca^{2+}imaging

All imaging experiments were performed on a digital epifluorescence imaging system (WinFluor, J. Dempster, University of Strathclyde) mounted on a Nikon Eclipse 2000 microscope using a 20× objective. Hippocampal cells (DIV 10–14) were loaded with Fluo-4 AM (5 μM, 45–60 min, room temperature) prior to experiments. Experiments were performed on cultures continually perfused (1–2 mL min^{-1}) with HEPES-buffered saline (HBS), with the following composition (in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, D-glucose10, pH 7.4, 310 mOsm, at room temperature. All compounds investigated were added via the perfusate. Data were calculated as changes in fluorescence ratio and expressed as $\Delta F/F_0$.

2.7. Biological assays on cockroach preparations

2.7.1. Electromyographical recordings

Peripheral neurotoxicity was evaluated using the *in vivo* cockroach metathoracic-coxal adductor muscle preparation [15]. Animals were immobilized by chilling and mounted, ventral side up, in a Lucite holder covered with 1 cm soft rubber that restrained the body and provided a platform to which the metathoracic coxae could be firmly attached using entomologic needles. The left leg was then tied at the medial joint with a dentistry suture line and connected to a 1 g force transducer (AVS Instruments, São Carlos, SP, Brazil). The transducer was mounted in a manipulator which allowed adjustment of muscle length. The exoskeleton was removed from over the appropriated thoracic ganglion. Then, the nerve 5, which includes the motor axon to the muscle, was exposed and a bipolar electrode was inserted to provide electrical stimulation. The nerve was covered with mineral oil to prevent dryness and then stimulated at 0.5 Hz/5 ms (indirect stimuli), with twice the threshold. The preparations were allowed to stabilize for at least 20 min before the addition of BBV and the experiments carried out during 120 min. Isometric muscle tension was recorded, digitalized and retrieved using a computer based software AQCAD (AVS Instruments, São Carlos, SP, Brazil). Data were further analyzed using the software ANCAD (AVS Instruments, São Carlos, SP, Brazil). In this set of experiments the venom was injected into the animal third abdominal segment by means of a Hamilton syringe (20 μl final volume).

2.7.2. Electrophysiological recordings

For the *ex situ* recordings of the *extracellular of spontaneous neural compound action potentials* (SNCAP) *of cockroach leg*, male cockroaches were anesthetized by chilling during 5–7 min and the metathoracic leg was removed by cutting as closely as possible to the body ensuring that the coxa, femur, tibia, and tarsus remained intact. The leg was then carefully fixed by means of three Ag/AgCl needle electrodes in a Petri dish recovered with 10 mm Sylgard®. One of these electrodes was connected to the ground connector of the amplifier (Axoclamp 2B, Molecular Devices, USA) and the other to the indifferent (−) connector of the amplifier. The third electrode was placed in the femur and was the active recording electrode (+). The signals were recorded at a sampling rate of 1 kHz and digitalized using a digitizer model Digidata 1320A (Molecular Devices, USA). The action potentials were visualized, recorded and retrieved for later analysis in the computer-based software Clampex (Molecular Devices, USA). The potentials were analyzed using the software WinWCP (John Dempster, University of Strathclyde). Using this configuration the preparation could be used for at least 1 h without changing the physiological characteristics of the potentials. BBV (32 μg/g) was injected directly into the leg, underneath the cockroach cuticle using a Hamilton syringe (10 μl final volume). Potentials were analyzed using the automatic waveform measurement module of WinWCP. A series of standard measurements on the digitized signals including, basic amplitude and duration measurements (e.g. peak, amplitude, average, area, rise time, and decay time) were made on each record in presence of BBV or saline. The analysis of the signal amplitude was performed as relative to the zero level using the following formula $\gamma_{peak} = Max_{i=1\ \ldots\ n}(a(i) - a_z)$ where $\gamma_{peak} = Max_{i=1\ \ldots\ n()}$ implied an algorithm to find the maximum value within a set n samples, $a(i)$. The temporal analysis of the potentials including the area of the signals was verified by applying the following formulas:

$$\gamma_{avg} = \frac{S}{n}\sum_{i=i_0}^{i_0+n-1}(a(i) - a_z) \quad \text{A}$$

$$\int_{\gamma} = S\sum_{i=0}^{n}(a(i) - a_z)dt \quad \text{B}$$

where, in A) i_0 is the first sample and n is the number of samples in the block. S is the scaling factor. a_z is the absolute zero level compared to the a_i which is taken by the absolute initial level. At B) the area enclosed by the signal and the zero level of the signal waveform is the integral γ and dt is the interval of time.

2.8. Statistical analysis

Data were expressed as means ± standard deviation (SD). Each experiment was repeated at least three times. For data comparisons between means of two different experimental groups we employed Student "*t*" test. When data from more than two experimental groups were analyzed, ANOVA was employed followed by Tukey (all groups were compared with each other) or Dunnet (the groups were compared with a positive control or saline) *post hoc tests*. All statistical analyses were performed using the Graphpad Prism 6.0 (Software Inc., San Diego, CA). The values were considered significantly different when $p \leq 0.05$.

3. Results

3.1. BBV shows in vivo effects on cockroach nerve-muscle preparations

To analyze the effect of BBV on cockroach peripheral nervous

system (PNS), we used the *in vivo* metathoracic coxal-adductor nerve-muscle preparation. The administration of insect saline alone did not interfere with the neuromuscular responses during 120 min recordings (n = 6) (Fig. 2). The injection of BBV at different concentrations (32, 64 and 128 μg/g of animal weight), induced a progressive dose-dependent neuromuscular blockade in 120 min recordings. In all concentrations assayed the twitch tension started to decrease after 70 min. At the dose of 32 μg/g of BBV, the twitch tension decreased to 50 ± 18% after 70 min and to 70 ± 4% after 120 min ($p < 0.01$ and $p < 0.001$, n = 6) (Fig. 2A). At 80 and 120 min, the dose of 64 μg/g reduced the twitch tension to 50 ± 19% and 70 ± 20%, respectively ($p < 0.01$ and $p < 0.001$, n = 6) (the representative trace of this experiment can be seen at Fig. 2A). In contrast, when the highest dose of BBV (128 μg/g) was assayed, there was only a 40 ± 11% ($p < 0.05$, n = 6) inhibition of the twitches after 120 min (Fig. 2A). In fact, the dose of 128 μg/g of BBV did not reach 50% inhibition of twitches when tested up to 120 min, while the doses of 32 and 64 μg/g took around 70 min to reach this level of inhibition ($p < 0.01$, n = 6) (Fig. 2C). Of note, at the end of 120 min all BBV-injected animals showed signs of spasticity (data not shown). With 64 and 128 μg/g of BBV there was a significant increase in the rise time of the twitches ($p < 0.01$ and $p < 0.001$, respectively, n = 6, Fig. 3).

To verify the possible involvement of sodium channels in the insect neuromuscular blocking activity of BBV, we carried out some protocols using TTX (3 ng/g of animal weight). In this set of protocols the assay of TTX alone induced a maximum inhibition of muscle twitches of 12 ± 14%, in 120 min recordings ($p < 0.05$, n = 6). When BBV (32 μg/g) was administered 60 min after a previous injection of TTX 3 ng/g, there was a complete prevention of the venom blocking activity (n = 6, $p < 0.05$ compared to the control BBV alone, results not shown).

3.2. BBV decreases the frequency of SNCAPs in cockroach legs

The recordings of SNCAPs of *Nauphoeta cinerea* legs showed that BBV (32 μg/g) induced a significant decrease in the frequency without affecting the amplitude of the potentials, in 60 min recordings (Fig. 4). Thus, the mean amplitude and frequency for the control saline were 0.59 ± 0.07 mV and 82 ± 3 min^{-1}, respectively. When the venom was assayed there was a slight increase in the amplitude (0.76 ± 0.06 mV) and a significant decrease in the frequency (36 ± 1.3 min^{-1}) (n = 6, $p < 0.05$ compared to the control saline) (Fig. 4A and C). The control parameters of the rise time, the decay time, duration and area in saline conditions were (0.66 ± 0.012 ms, 3.1 ± 0.05 ms, 1.93 ± 0.12 ms and 0.24 ± 0.01 mV ms). However, after the treatment with BBV (32 μg/g) only the area of the SNCAP was significantly changed to 0.79 ± 0.05 mV ms in 60 min recordings (Fig. 4B and D).

3.3. BBV affects rat hippocampal cell viability

The incubation of primary rat hippocampal cells with BBV (4 μg/mL) for 5 min did not change the percentage of dead cells as compared to the saline controls (6 ± 1.6%, n = 4, and 5 ± 1.8%, n = 7, respectively, $p = 0.3195$). However, when the cells were incubated for the same time with the positive control, 0.01% sodium hypochlorite, 100% of the cells died (n = 7).

3.4. BBV increases calcium influx in rat hippocampal cells

By using calcium images to monitor intracellular calcium levels

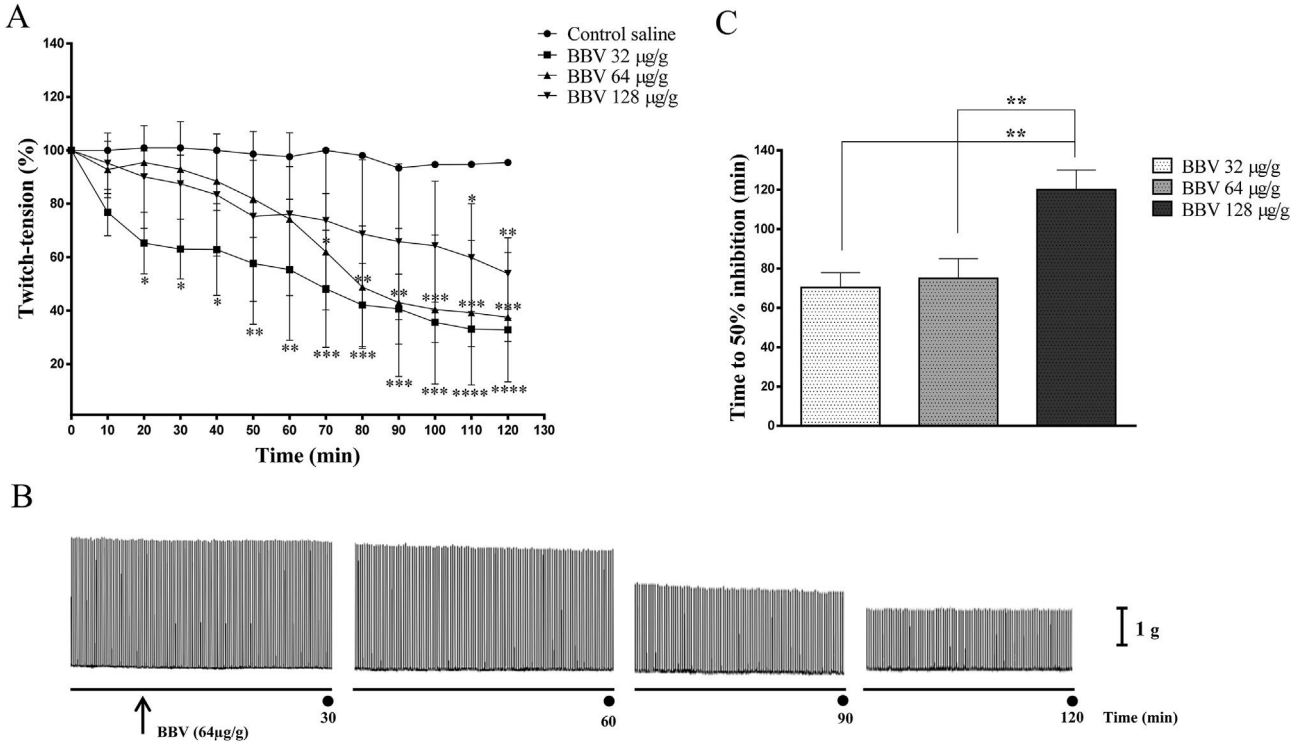

Fig. 2. Neuromuscular blockade induced by BBV on the *in vivo* cockroach coxal-adductor methatoracic nerve-muscle preparation. (A), shows the means ± standard deviation (SD) of the dose-response effect of BBV (32, 64 and 128 μg/g of animal weight), in 120 min recordings. The statistical analysis was performed by Two-Way Anova followed by Dunnett test as *post hoc*. (B) shows a representative trace of the time course of cockroach muscle strength under BBV (64 μg/g) treatment. (C), shows the time to 50% inhibition of the muscle twitches. The statistical analysis was performed by One-Way Anova followed by the Tukey test. The results are expressed as mean ± standard deviation (SD). $^{*}p \leq 0.05$, $^{**}p < 0.01$, (n = 6).

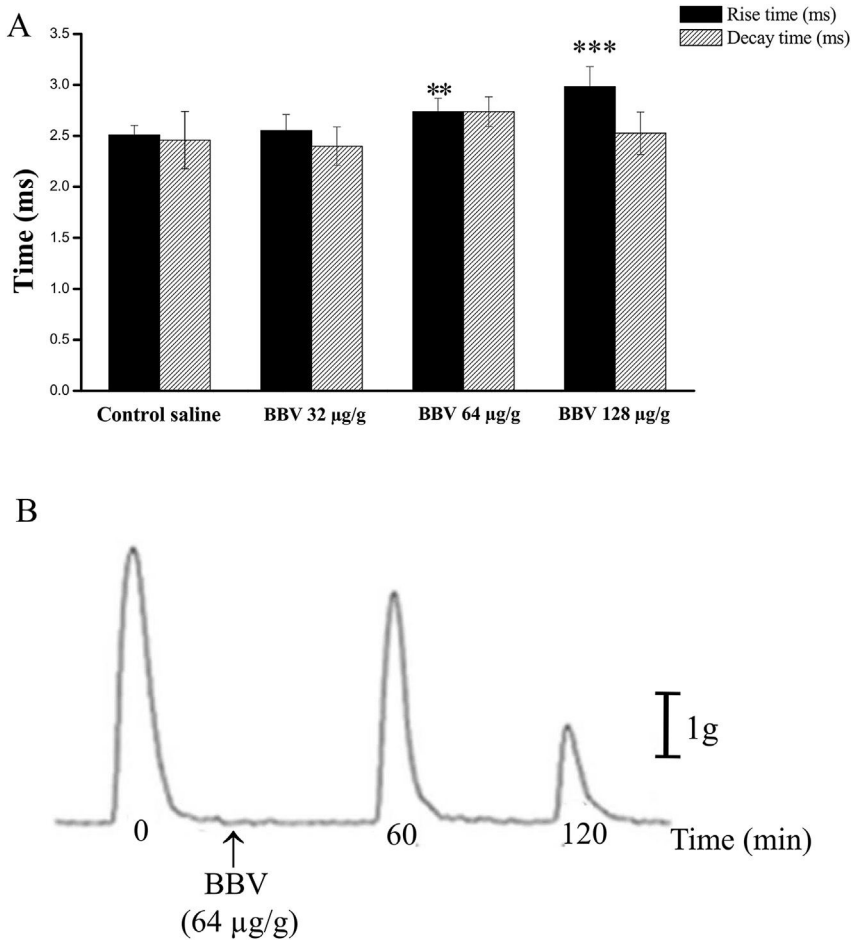

Fig. 3. Kinetics of *in vivo* cockroach coxal-adductor methatoracic nerve-muscle preparations treated with BBV. (A) shows the mean rise and decay time during 120 min recordings, in presence of BBV (32, 64 and 128 µg/g of animal weight). (B), shows the magnification of individual twitches of an animal treated with BBV (64 µg/g). The statistical analyses were performed by One-Way Anova followed by the Dunnett test. The results are expressed as mean ± standard deviation (SD). $^{**}p < 0.01$ and $^{***}p < 0.001$, (n = 6).

($[Ca^{2+}]_i$), BBV (4 µg/ml) induced a pronounced rise in neuronal $[Ca^{2+}]_i$ (250 ± 9% peak increase, n = 3, $p < 0.001$, Fig. 5B and C). Interestingly, depolarizing the neurons by applying high potassium (35 mM) after BBV infusion did not further increase significantly the fluorescence induced by the venom (Fig. 5C). However, after a continuous 10 min wash of the cells with HBS, the fluorescence returned to the basal level (Fig. 5C). In this set of experiments the treatment of the cells with 4-AP (250 µM) induced a significant rise in $[Ca^{2+}]_i$ (6 ± 0.9, n = 35 cells from three cultures) (Figs. 6B and 7). When the cells were treated with glutamate (GLU, 100 µM), there was also an increase in the $[Ca^{2+}]_i$ (5.4 ± 1.5, n = 36, Figs. 5C and 6). The treatment of the cells with TTX (50 µM) before infusing GLU or TTX prevented significantly ($p < 0.05$) the increase in $[Ca^{2+}]_i$ of both GLU and BBV (0.4 ± 0.15 and 0.4 ± 0.3, respectively, Figs. 6D and 7). In contrast, the co-infusion of 4-AP with BBV could not prevent the $[Ca^{2+}]_i$ increase caused by BBV (n = 30, $p < 0.0001$ compared to BBV alone, Fig. 7).

4. Discussion

The results clearly show that BBV induced pronounced alterations both on cockroach peripherical nerves and on rat hippocampal cells, selected as model of the effects on mammalian central nervous system (CNS).

Similarly to some Buthidae venoms, BBV affects insect nerve muscle preparations in a slow and progressive way [16,17]. Indeed, under our experimental conditions both repetitive twitches, during the onset of venom activity, and final spastic paralysis of the cockroach muscles were observed. The fact that BBV also induced a significant increase in the area of SNCAP together with the venom myographic profile, could indicate a modulation of the gating properties of some class of ion channels. In our *in vivo* experiments, the previous application of a sublethal dose of TTX in the cockroaches, significantly prevented the blockage induced by BBV on the neuromuscular twitches. This result suggests a putative activity of BBV on voltage gated sodium channels (VGSC). Thus, although this action on VGSC is not typical of non-Buthidae venoms, our results support its involvement in the BBV effect on insect neuromuscular preparations [18]. As mentioned in the introduction of this work, scorpion neurotoxins that modulate VGSC are classified in two categories: α- and β-Toxins. α-Toxins slow voltage-gated sodium channels inactivation by binding in site 3 of the channel, therefore prolonging the action potentials [19,20]. β-Toxins, bind in the receptor site 4 to shift the sodium channels voltage dependence of activation to a more negative membrane potentials [21,22]. It is also worthy of note that α-toxins are exclusively found in Old World scorpions [23], thereby β-type toxins would be strong candidates for the sodium-channel pharmacological modulation induced by BBV.

In addition, the large amount of chemical pesticides employed in agriculture damage not only insect pests, but also beneficial insects, and other living organisms from the ecosystem [24]. It has

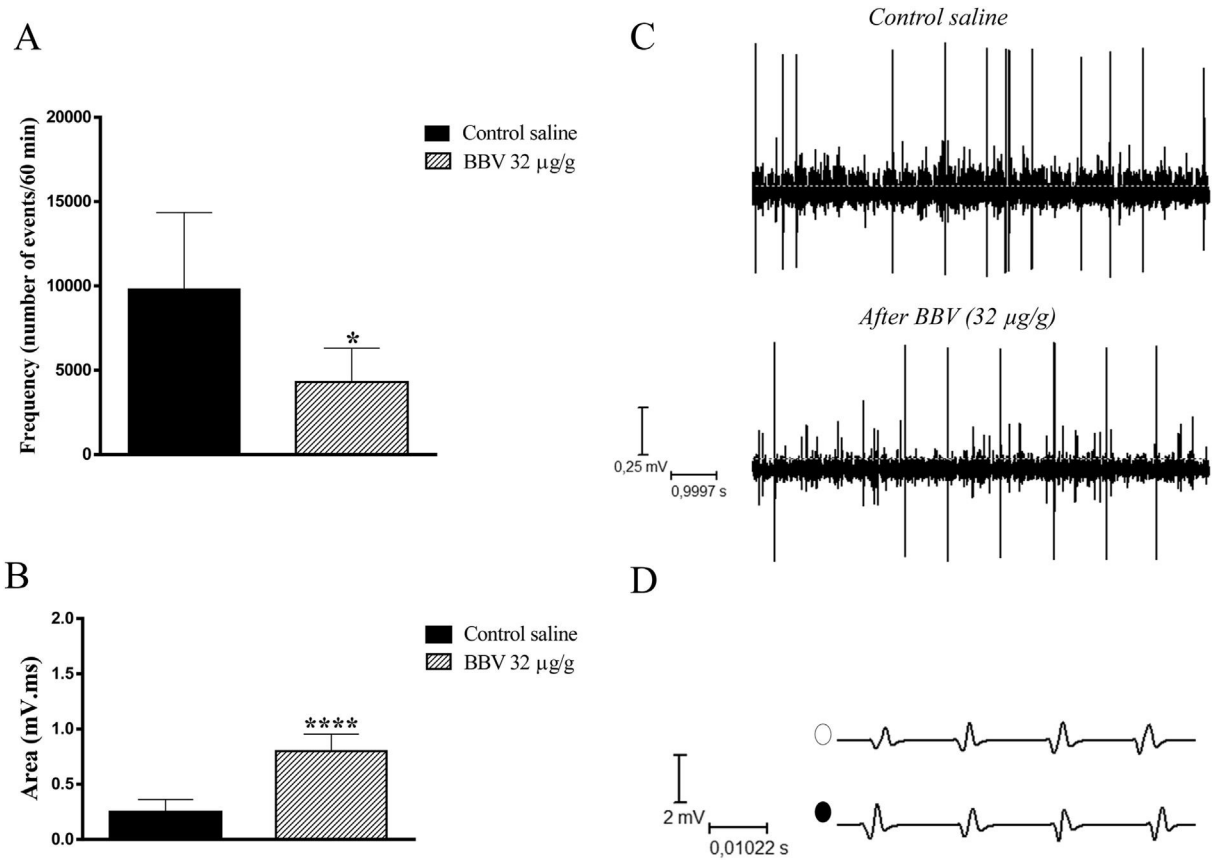

Fig. 4. Depressant activity induced by BBV on the frequency of the SNCAP. (A) displays the number of events during 60 min recordings. (B) shows the mean area of the SNCAP during 60 min recordings. (C), representative traces of the SNCAP under control saline conditions and during the onset of BBV activity. (D) shows the average recordings of SNCAP comparing the control saline (○) and the BBV treatment (·). On A and B, data are expressed as mean ± standard deviation (SD), and the statistical analyses were performed by Student's "*t*" test: $^{*}p < 0.05$ (n = 6); $^{****}p < 0.0001$ (n = 6).

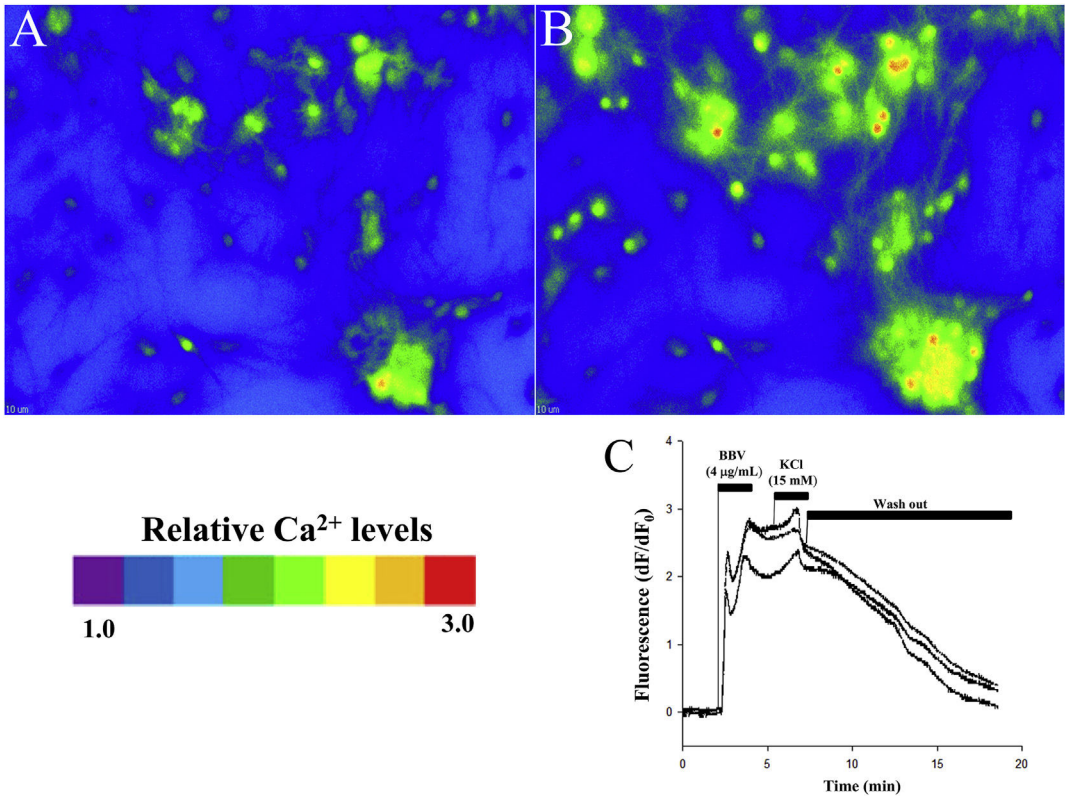

Fig. 5. Increase of Ca^{2+} influx ($[Ca^{2+}]_i$) caused by BBV in primary cultures of rat hippocampal cells. Panels (A) and (B) show representative images of cells loaded with Fluo-4/AM during base-line and during 5 min of BBV perfusion, respectively. (C), the graph shows the change in Fluo-4/AM fluorescence emission ($\Delta F/F_0$) induced by the BBV (4 µg/ml) application.

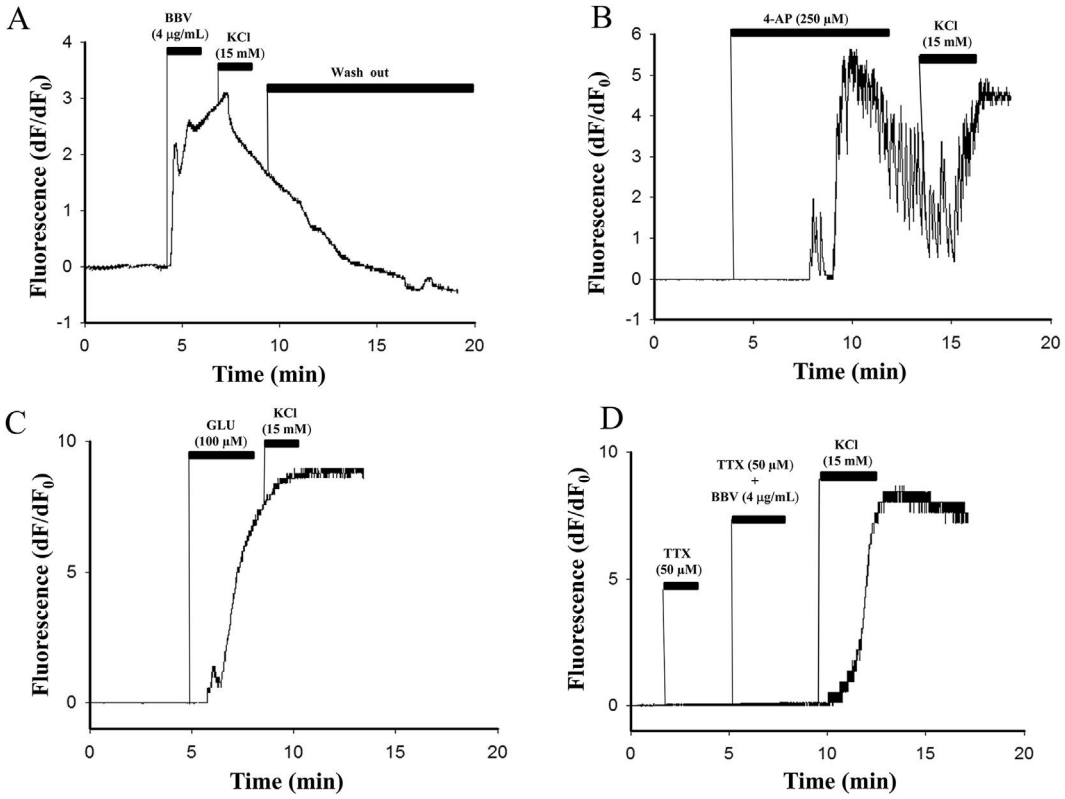

Fig. 6. Preventive effect of TTX on the increase of $[Ca^{2+}]_i$ induced by BBV in primary cultures of rat hippocampal cells. (A), a typical trace of $[Ca^{2+}]_i$ during BBV (4 µg/ml) infusion. (B), representative trace of the effect of 4-AP (250 µM) on $[Ca^{2+}]_i$. (C), the graph shows the recording of Fluo-4/AM ratio ($\Delta F/F_0$) induced by the application of GLU (100 µM), an AMPA/kainate sodium channel activator. (D), traces of calcium fluorescence in neurons treated with TTX (50 µM) before BBV (4 µg/ml) treatment.

been shown that the skeletal musculature of different animals is affected by distinct toxins present in the crude venoms, the so-called mammalian, crustacean and insect toxins [8]. Nevertheless, in scorpion venoms, some neurotoxins present a selective activity against specific insect pests, without affecting the beneficial ones or mammalians [25,26]. For this reason, the effect of BBV on the neuromuscular activity in insects reveals a potential applicability of components from this venom as potential natural pesticides [25].

Further evidence that the activity of BBV in biological membranes involves sodium channels modulation comes from our experiments on $[Ca^{2+}]_i$ in rat hippocampal cells. In mammalian neurons, calcium influx is a well-coordinated process, which has a close relationship with the sodium currents [26]. The physiological process starts with the membrane depolarization induced by AMPA receptors activated by the neurotransmitter GLU, resulting in increased sodium influx [27]. Rapid adjustments of $[Ca^{2+}]$ are then made by Na^+/Ca^{2+}–K^+ and Na^+/Ca^{2+} exchangers (NCX), while the fastest known Ca^{2+} signaling proteins are the voltage-gated Ca^{2+}channels CaVs [28]. During an action potential, the CaVs rapidly increase the $[Ca^{2+}]_i$, triggering the opening of ryanodine

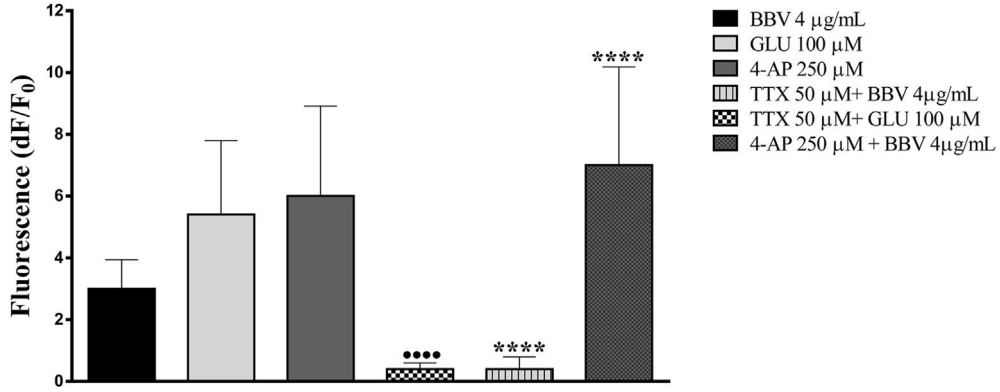

Fig. 7. Comparison of BBV effect on $[Ca^{2+}]_i$ in primary cultures of rat hippocampal cells with different pharmacological treatments. In the graph each bar represents the fluorescence of cells loaded with Fluo-4/AM (5 µM) from three different cultures. Results were expressed as mean ± standard deviation (SD) Statistical significance was determined by One-way Anova followed by Tukey test as *post hoc* to compare cells infused with BBV with those infused with TTX+BBV, and cells infused with 4-AP+BBV (****$p < 0.0001$, n = 30), and by using Student's t-test to compare cells infused with GLU with cells infused with TTX+GLU, or cells infused with 4-AP with cells infused with 4-AP+BBV (… $p < 0.0001$, n = 30).

and inositol triphosphate (IP3) receptors spanning the endoplasmic reticulum membrane. As a result, there is a further increase in the $[Ca^{2+}]_i$, enabling the fusion of vesicles containing transmitters to the plasma membrane [29]. Then, NCX and the Ca^{2+}-ATPase-pump rapidly reduce the $[Ca^{2+}]_i$ to basal levels [29].

There is evidence linking alterations in Ca^{2+} signaling and neuronal apoptosis [30]. In particular, in the CNS, excessive GLU in synaptic terminals can lead to Ca^{2+} overload via the overactivation of ionotropic NMDA receptors, establishing a process of excitotoxicity [31]. However, under our experimental conditions the same concentration of BBV which increased intracellular $[Ca^{2+}]_i$ by 200% did not provoke cell death in the primary cultures of hippocampal cells, as demonstrated by the Trypan Blue exclusion method. This make BBV components interesting candidates to be tested and compared to the sodium channel modulators phenytoin, carbamazepine, lamotrigine, and lidocaine regarding their therapeutic potential to treat diseases such as epilepsy, neuropathic pain, and arrhythmias [32]. Moreover, their target specificity to ion channels and relatively high biological stability make peptides derived from natural venoms molecular prototypes with especial interest for the development of medicines to treat ion channel-related diseases [33]. Thus, our results also reinforce the potential of BBV as a source of novel therapeutic molecules.

Here we show that TTX, a classical sodium channel blocker, abolished the BBV-induced $[Ca^{2+}]_i$ rise in hippocampal cells. We also tested the effect of BBV on $[Ca^{2+}]_i$ in the presence of the voltage-gated K^+-channels blocker 4-AP. This protocol was designed on considering that non-Buthidae venoms usually possess low amounts of sodium channel specific toxins and high number of potassium channel counterparts [34]. Since the co-infusion of BBV with 4-AP did not alter the $[Ca^{2+}]_i$ caused by the aminopyridine alone, we suppose that voltage-gated K^+-channels may not be involved in the effects of BBV on $[Ca^{2+}]_i$ [35]. However, we cannot completely discard that potassium channels modulators are not present in the venom. Altogether, our data suggest that the biological activity of BBV in mammalian CNS neurons and in insect PNS involve mainly the activation of voltage-gated sodium channels as molecular targets. The lack of BBV functional selectivity between different species reinforces the possible presence of a specific class of β-toxins, which are active on both insect and mammalian Nav channels [22]. A direct modulation induced by BBV on sodium channels activity could also explain the pain-related events described by the patients stung by this scorpion. Therefore, it is well established that hyperexcitability and/or increased baseline sensitivity of primary sensory neurons can lead to abnormal burst activity associated with pain [36,37]. However, we cannot rule out that the increase of calcium influx in hippocampal cells could, at least in part, result from a direct modulation of GLU release, leading to membrane depolarization by increasing Na^+ influx and calcium concentration. Accordingly, effects of alpha- and beta-Na^+ channel scorpion toxins on GLU release was previously reported [38]. It would be of interest to carry out further studies to identify and isolate the neuroactive compounds of BBV.

5. Conclusions

BBV shows powerful biological activity on both insect and mammalian models. The modulation of both muscle twitches and spontaneous compound action potential time course in cockroaches and the massive Ca^{2+} influx in rat hippocampal cells reveals a direct activity of the venom toxins on Na^+ channels. The identification of the mechanisms involved in the BBV neurotoxicity, together with the isolation of active components of the venom will open new avenues for future biotechnological and clinical applications including the preparation of novel pesticides and the development of innovative therapies.

Conflict of interests

The authors declare no conflict of interests related to this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cbi.2016.08.008.

Transparency document

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II. Artigo Publicado

Stürmer et al. 2014

Participação no desenho e execução dos experimentos que avaliaram a modulação da neurotransmissão dopaminérgica em baratas sob a influência de doses subletais do organofosforado trichlorfon.

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Modulation of dopaminergic neurotransmission induced by sublethal Doses of the organophosphate trichlorfon in cockroaches

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ABSTRACT

Organophosphate (OP) insecticides have been used indiscriminately, based on their high dissipation rates and low residual levels in the environment. Despite the toxicity of OPs to beneficial insects is principally devoted to the acetylcholinesterase (AChE) inhibition, the physiological mechanisms underlying this activity remain poorly understood. Here we showed the pharmacological pathways that might be involved in severe alterations in the insect locomotion and grooming behaviors following sublethal administration of the OP Trichlorfon (Tn) (0.25, 0.5 and 1 μM) in *Phoetalia pallida*. Tn inhibited the acetylcholinesterase activity (46 ± 6, 38 ± 3 and 24 ± 6 nmol NADPH/min/mg protein, $n=3$, $p<0.05$), respectively. Tn (1 μM) also increased the walking maintenance of animals (46 ± 5 s; $n=27$; $p<0.05$). Tn caused a high increase in the time spent for this behavior (344 ± 18 s/30 min, 388 ± 18 s/30 min and 228 ± 12 s/30 min, $n=29$–30, $p<0.05$, respectively). The previous treatment of the animals with different cholinergic modulators showed that pirenzepine > atropine > oxotremorine > *d*-tubocurarine > tropicamide > methoctramine induced a decrease on Tn (0.5 μM)-induced grooming increase, respectively in order of potency. Metoclopramide (0.4 μM), a DA-D_2 selective inhibitor decreased the Tn-induced grooming activity (158 ± 12 s/30 min; $n=29$; $p<0.05$). Nevertheless, the effect of the selective DA-D_1 receptor blocker SCH 23390 (1.85 μM) on the Tn (0.5 μM)-induced grooming increase was significative and more intense than that of metoclopramide (54 ± 6 s/30 min; $n=30$; $p<0.05$). Taken together the results suggest that a cross-talking between cholinergic M1/M3 and dopaminergic D1 receptors at the insect nervous system may play a role in the OP-mediated behavioral alterations.

1. Introduction

Organophosphate insecticides (OP) derived from phosphoric or thiophosphoric acid include parathion, malathion, diazinon, fenthion, chlorpyrifos (Nishizawa 1960; Fest and Schmidt 1982; Medegela et al. 2010), and trichlorfon (Tn) among others. Tn (metrifonate or dimethyl 2,2,2-thrichloro-1-hydroxyethyl phosphonate) inhibits acetylcholinesterase (AChE) due to its ability to phosphorylate its serine hydrolase at its esterasic active site. AChE inhibition leads to accumulation of acetylcholine at the cholinergic junction from nerve tissue and effector organs, producing acute effects at the muscarinic and nicotinic receptors and at the central nervous system (CNS) (Torres-Altoro et al. 2011; Cummings and Ringman 1999). Tn was first registered in the United States in 1955, and nowadays is a worldwide diffused systemic insecticide with a variety of domestic and agricultural applications (EPA 2006). Li and colleagues (2011) (Li et al., 2011) have shown that the dissipation rates of Tn on soil and cabbage are about 90% in five days, suggesting that Tn is safe when applied at the recommended dosage. Although the dissipation and degradation rates of OP are high, the potential effect of residual amounts of these insecticides in the surrounding environment is still a matter of concern. In this way, even if their concentrations are low in the environment (Gupta et al., 2012), the reminiscent activity of these insecticides are amenable to induce long-term behavioral alterations in insects (de Castro et al., 2013; He et al., 2013, Neuman-Lee et al., 2013).

During the last two decades, the effects of OP on beneficial arthropods have been the subject of an increasing number of

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studies, and their potential effects have been reviewed several times (Croft, 1990; Haynes, 1988; Thompson, 2003; Desneux et al., 2007). In addition to the direct mortality, the effects of sublethal doses of OP on arthropod physiology and behavior must be considered in a complete analysis of their impact, although these behavioral alterations are difficult to study or predict (Desneux et al., 2007). While evaluations associated with the direct effects of pesticides on natural enemies are important, the indirect or delayed effects of pesticides are actually more relevant because they provide information on the long-term stability and overall success of a biological control program when attempting to integrate the use of pesticides with natural enemies (Cloyd, 2012). Therefore, these behavioral alterations could increase the chances of beneficial insects being preyed upon, and thus have the potential for serious ecological consequences through trophic transfer.

Early studies of Guez and colleagues (2005) have shown important alterations induced by sublethal doses of OPs on foraging behavior in honeybees. Castro et al. (2013) and Campos et al. (2001) also have described the effect of sublethal concentrations of OPs on mobility parameters in pentatomid stinkbugs, a natural predator of defoliating caterpillars, and in earwigs, a predator of armyworms, respectively. Although the authors have identified the behavioral alterations derived from the insect OP sublethal intoxication, none of these studies have deeply addressed specific neurophysiological mechanisms. In this context, any indirect effects, which are sometimes referred to as sub-lethal, latent, or cumulative adverse effects may interfere with the physiology and behavior of natural enemies by inhibiting longevity, fecundity, reproduction, development time, behavior (mobility, searching, foraging, and feeding), predation and/or parasitism, prey consumption, emergence rates, and/or sex ratio (Cloyd, 2012).

Despite the role of dopamine in modulating behavior in insects has been extensively studied, the influence of cholinergic modulators (e.g. sublethal doses of OP) on this parameter is still not well understood. Furthermore, most neurons in the nervous system apparently contain and release more than one neurotransmitter or neuromodulator. Because of this, it is thought that such co-transmission should be considered as a rule instead of an exception (Trudeau, Gutierrez, 2007). In this aspect, cockroaches are primitive insects in which most of functional systems are fairly unspecialized and for this reason have been used not only to understand the physiological aspects between insect and chemical compounds, but also as an important model for biomedical research (Stankiewicz et al. 2012).

Here we sought to investigate the role of sublethal doses of Tn in modulating biochemical and neurophysiological parameters affecting insect behavior, using cockroaches (*Phoetalia pallida*). We have also compared the in vitro and in vivo effects of Tn on the cholinergic and dopaminergic systems of the insects. However, as far as of our knowledge, there is no previous work demonstrating the extra-cholinergic pathway involved in the modulation of insect behavior by organophosphates. Thus, the rationale for such study in cockroaches is not restricted to the pharmacology of OP itself, but may contribute for the elucidation of the unknown mechanisms involved in the recent behavioral alterations of other beneficial insects like honey bees.

2. Materials and methods

2.1. Experimental animals

All experiments were performed on adult male *P. pallida* cockroaches (3–4 months after adult molt). The animals were reared at laboratory conditions of controlled temperature (22–25 °C) on a 12 h:12 h L:D cycle. All cockroaches were provided with water and dog food ad libitum. Prior to analysis of biochemical and neurophysiological parameters, the maximum sublethal dose of Tn was determined in cockroaches essentially as described by Kagabu et al. (2007).

2.2. Drugs and chemicals

Tn, atropine, tyramine, methoclopramide, pyrenzepine, methoctramine, d-tubocurarine, oxothremorine, tropicamide, nifedipine, and SCH23-390 were obtained from Sigma-Aldrich (USA) and were freshly prepared in HEPES saline of the following composition (in mMol l^{-1}): NaCl, 214; KCl, 3.1; $CaCl_2$, 9; sucrose, 50; HEPES buffer, 5 and pH 7.2 (Wafford and Sattelle 1986). All drugs were administered at the third abdominal hemocoel segment, at a final volume of 20 μL by means of a Hamilton syringe. Experiments were performed at controlled room temperature (22–25 °C) in accordance with the guidelines of the National Counselor to Control Animal Experimentation-CONCEA.

2.3. Assay for determining sublethal doses

The insecticidal assay against adult *P. pallida* was conducted in an adaptation of the assay described by Kagabu et al. (2007). The average weight of the insects (MW) was determined by weighting three hundred adult animals. Thus, the MW was found to be 0.55 g, which was used to calculate the actual doses to be used. Tn was dissolved in HEPES saline and injected into the second abdominal segment of *P. pallida* at concentrations ranging from 0.25 to 4 μM, in a final volume of 20 μL by means of a Hamilton syringe. Three insects were used to test each dose of trichlorfon and after treatment they were kept in a plastic bottle at 22–25 °C for 24 h with free access to water and food. The minimum dose that killed all the three insects in 24 h was considered as the minimum lethal dose (MLD). Paralyzed insects were also considered as if they were dead. In our experimental condition, it was found that the MLD was 2 μM, and the 4 μM concentration was able to kill all the three animals in 24 h. Thus, in the present work doses below the MLD were chosen for conducting the behavioral assays.

2.4. Assay for cholinesterase activity

The in vitro activity of AChE was evaluated according to the assay described by Ellman et al. (1961) modified by Franco et al. (2009). The whole amount of protein was measured according to Bradford (1976). Three cockroaches were injected with Tn (0.25, 0.50, and 1 μM) and after 6 h were anesthetized by chilling at −5 °C and their brains were collected following the removal of the cuticle. The tissue was mixed with 750 μL of Kpi buffer pH 7.0, centrifuged (500 rpm/5 min/4 °C) and 400 μL of supernatant was collected. Fifty μL of the supernatant was added to 50 μL of 50 mM DTNB, 500 μL of Kpi (pH 8.0) and 2.5 μL of acetylthiocoline. The reaction was measured after 1 min at 412 nm using a UV-Visible Spectrophotometer (model Evolution 60 S, Thermoscientific, New Hampshire, USA). The results were analyzed by the software VISION lite (Thermoscientific).

2.5. Video-mounting apparatus for biological assays

For each specific biological assay, the activities were recorded during 30 min by using a video-camera (Panasonic coupled to a 50 mm Karl-Zeiz lens) connected or not to an eyepiece of microscopy (Olympus, model SZ51, Germany). The camera had a frame-by-frame (60/s) and was connected to a PC (Infoway, ItauTec, Brazil). Video movies were later analyzed using a HD Writer AE 2.6 T system (Panasonic) with variable speed control.

2.6. Biological assays

2.6.1. Behavioral assays

For general behavioral study, animals were placed in a demarked open-field arena with a video camera mounted overhead.

2.6.1.1. Sustained locomotor activity. In order to analyze walking maintenance, a behavior paradigm based on the methodology described by Gal and Libersat (2008), namely the forced swimming test was applied. A continuous stressful stimulus producing strong, stereotypic aversion responses was created by water immersion (Cocatre-Ziligien and Delcomyn 1990; Gal and Libersat 2008). Swimming in a modified forced swimming test was induced by placing the cockroaches in an opaque pool (25 cm in diameter) filled with water to a height of 10 cm, maintained at 25 °C. The insects were monitored for 1 min period using a video camera. The swimming behavior was based on the time the insects kept in motion.

2.6.1.2. Grooming activity. The grooming behavior of cockroaches was monitored in an opaque plastic box (29 cm x18 cm x 13 cm) with a clear plastic cover (Weisel-Eichler et al. 1999) and was recorded with a camera for later analysis of motion time. The time of continuous grooming in seconds was measured for a 30 min

period immediately following treatment. Animals had never been placed in the testing box previously, and it was therefore a novel environment in all cases. The temperature in the testing room was maintained at 25–30 °C. Testing was performed 2–8 h after the beginning of the light cycle. Control cockroaches were induced to groom by handling (Weisel-Eichler et al. 1999).

2.7. Data statistical analysis

The results were expressed as the mean ± SEM and were analyzed using analysis of variance (Two-Way ANOVA), followed by the Student "t" test as a *post hoc*. A p-value ≤ 0.05 indicated significant differences between the groups. Statistics were calculated and graphs produced using the Software OriginPro 8.6 (OriginLab Corporation, MA, USA).

3. Results

3.1. Effect of sublethal doses of trichlorfon on brain AChE activity

The analysis of AChE activity from cockroach brain homogenates before and after injecting different sublethal concentrations of Tn revealed a time and dose-dependent enzyme inhibition. The enzyme activity of the control values, before the injection of Tn with saline was 60 ± 14 nmol NADPH/min/mg protein. When 0.25 μM of Tn was injected, the AChE activity dropped to 46 ± 6 nmol NADPH/min/mg protein ($n=3$; $p>0.05$). When 0.5 μM of Tn was injected there was a more pronounced decrease in the AChE activity (38 ± 3 nmol NADPH/min/mg protein; $n=3$; $p<0.05$ unpaired test t) compared to saline. A further increase in the Tn concentration (1 μM) resulted in an even more marked inhibition of AChE (24 ± 6 nmol NADPH/min/mg protein; $n=3$; $p<0.05$) (Fig. 1).

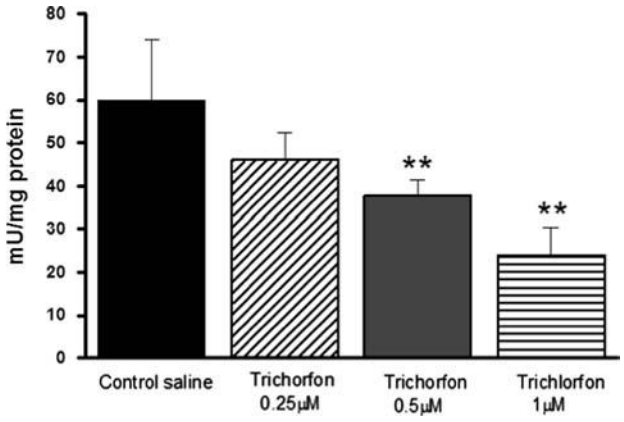

Fig. 1. Graph of acetylcholinesterase inhibition in total brain homogenate ($n=3$ for each dose) after 6 h exposure to trichlorfon (0.25; 0.5; 1 μM). Data were expressed as mU/mg protein. $^{**}p<0.01$ in comparison to saline control.

3.2. Effect of sublethal doses of trichlorfon on cockroach neurolocomotor activity

To analyze the influence of Tn on walking maintenance we used the forced swimming test. Control and organophosphate-injected individuals were immersed in a tank filled with water and the time of spontaneous active swimming trial was recorded during 1 min. All control individuals initiated swimming upon immersion, quickly reaching the wall and continuing to swim rigorously close to the rim. Overall, control subjects spent 30 ± 8 s ($n=30$) of the 1 min trial actively swimming. Swimming behavior normally stopped at 1 min, but the movement of the antennae remained in an exploratory manner, while the animal floated passively in the water surface. In this state, the application of tactile stimuli evoked another active swimming. This same vigorous wall-oriented swimming immediately upon immersion occurred only in 66% of all the insects treated with Tn, regardless the concentration. Therefore, there was not a dose-dependent effect within the three concentrations of Tn assayed. Nevertheless, in marked contrast to members of the control group, the swimming time in all the Tn-treated individuals increased, while the antennal movements were the same as the controls. Thus, in the cockroaches injected with 0.25 μM of Tn the mean time of active swimming increased to 42 ± 5 s ($n=28$; $p>0.05$). Surprisingly, the ones submitted to a dose of 0.5 μM did not present any change of swimming time as compared to the saline controls (34 ± 1 s; $n=30$; $p>0.$). However, when the concentration of 1 μM was injected into the insects, it was observed a maximum increase of the swimming time (46 ± 5 s; $n=27$; $p<0.05$) (Fig. 2).

3.3. Effect of sublethal doses of trichlorfon on grooming activity

In saline-injected cockroaches the mean time of continuous grooming was 75 ± 8 s/30 min ($n=28$). We found that the manipulation and the introduction of the rod of the syringe did not interfere with the normal behavior (87.5 ± 12 s/30 min; $n=28$; $p>0.05$).

However, when Tn was injected at different concentrations, there was a marked increase in grooming activity, despite it was not dose-dependent. Thus, the injection of 0.25 μM of Tn induced a significant increase in grooming activity (344 ± 18 s/30 min; $n=30$; $p<0.05$). This parameter was incremented with the dose of 0.5 μM of Tn (388 ± 18 s/30 min; $n=29$; $p<0.05$). However, when the highest concentration of Tn (1 μM) was administered, there was also an increase in the grooming time (228 ± 12 s/30 min; $n=30$; $p<0.05$), but below the time spent by the insects injected with 0.25 and 0.5 μM of Tn (Fig. 3).

3.3.1. Effect of cholinergic modulators on trichlorfon-induced grooming activity

In order to investigate whether cholinergic receptors are involved in the effects of Tn on grooming behavior, the insects were treated with cholinergic antagonists/agonists for 10 min before Tn injections. The injection of 3 μM of atropine, a non-selective inhibitor of cholinergic muscarinic receptors, did not induce any significative alteration in the grooming time (76 ± 2 s/30 min) when compared to the saline controls (75 ± 14 s/30 min) ($p>0.05$, $n=29$, respectively). Thus, none of these drugs (1 μM of methoctramine, 4.5 μM of tropicamine, 1.5 μM of d-tubocurarine, 1 μM of methoctramine, 3 μM of oxotremorine, and 3 μM of pirenzepine) induced significative changes in the grooming behavior when injected alone into the insects (Table 1).

The pre-treatment of the insects with methoctramine, a selective inhibitor of the insect M_2 cholinergic receptor, did not change the effect of Tn (418.5 ± 15 s/30 min; $n=29$; $p>0.05$). However, the injection of the selective M_4-cholinergic antagonist tropicamine, partially prevented the increase in the grooming time caused by Tn (105 ± 11 s/30 min; $n=28$; $p<0.05$), while d-tubocurarine, a competitive blocker of neural and muscle-type ionotropic receptors, fully prevented this effect (85 ± 5 s/30 min; $n=29$; $p<0.05$). Pre-treatment with the non-selective inhibitor of muscarinic receptors atropine and the non-selective muscarinic agonist oxotremorine not only prevented the Tn effect, but also decreased the grooming activity below the time spent by the saline controls (30 ± 3 s/30 min; $n=30$; $p<0.05$ and 44 ± 12 s/30 min; $n=30$; $p<0.05$, respectively). In this line, the pre-treatment with pirenzepine, a selective M_1/M_3-muscarinic blocker in insects, also abolished the effect of Tn on grooming activity,

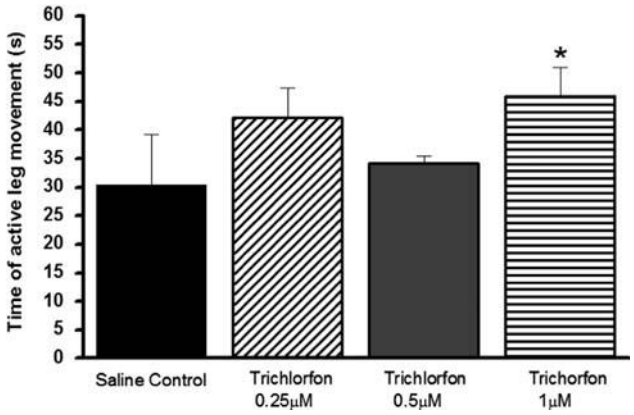

Fig. 2. Influence of different concentrations of trichlorfon (0.25; 0.5; 1 μM) on cockroach locomotor activity. The bars show the amount of time (in seconds) of constant swimming during 1 min recording. *$p < 0.05$ in comparison to saline control.

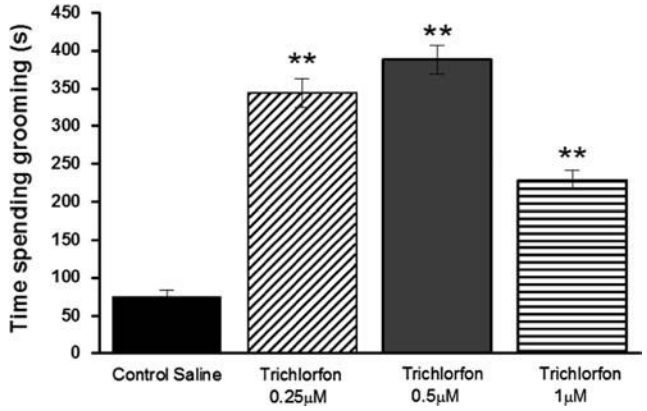

Fig. 3. Increase of grooming behavior by different sublethal doses of trichlorfon (0.25; 0.5; 1 μM). The grooming activity was recorded during 30 min and the results expressed as the total time of grooms in seconds. **$p < 0.01$ in comparison to saline control.

providing the lowest grooming time among all the drugs tested (15 ± 8 s/30 min; $n=30$; $p < 0.05$) (Fig. 4).

3.3.2. *Effect of dopamine modulators on trichlorfon-induced grooming activity*

Since dopamine (DA) activity is mediated by dopamine receptors at pre- and postsynaptic neuronal membranes (e.g. D_2 and D_1 receptors families), the protocols described below aimed to verify the influence of dopaminergic modulators on Tn-induced grooming activity. All drugs were administered 10 min prior treatment with Tn. Thus, when metoclopramide (0.4 μM), a DA-D_2 receptor antagonist, was injected before Tn (0.5 μM), there was a partial prevention of the Tn-induced grooming activity (158 ± 12 s/30 min; $n=29$; $p < 0.05$) (Fig. 5). Pre-treatment with SCH 23390 (1.85 μM), a selective DA-D_1 receptor blocker, provoked a significant inhibition of grooming levels, even below the control saline levels (54 ± 6 s/30 min; $n=30$; $p < 0.05$) (Fig.5). Since dopamine release depends on calcium influx at the nerve terminals in order to induce exocytose (Rozov et al. 2001), the voltage-gated calcium channel blocker nifedipine was also tested. Nifedipine (2.6 μM) alone extinguished the grooming activity (1 ± 0.35 s/30 min, $p < 0.05$, $n=30$) and the same was observed when it was applied prior Tn (0.5 μM)(1.66 ± 0.4 s/30 min; $n=29$; $p < 0.05$) (Fig. 5).

Table 1
Influence of different modulators on trichlorfon-induced grooming increase in cockroaches.

Treatment	Time of Grooming/s	Number of experiments	*Significance compared to control saline
Nifedipine (2.6 μM)	1.66 ± 0.4 s/30 min	$n=28$	*$p < 0.05$
Pirenzepine (3 μM)	15 ± 8 s/30 min	$n=30$	*$p < 0.05$
Atropine (3 μM)	30 ± 3 s/30 min	$n=30$	*$p < 0.05$
Oxotremorine (3 μM)	44 ± 12 s/30 min	$n=30$	*$p < 0.05$
SCH 23390 (1.85 μM)	54 ± 6 s/30 min	$n=29$	*$p < 0.05$
d-tubocurarine (1.5 μM)	85 ± 5 s/30 min	$n=29$	*$p < 0.05$
Tropicamide (4.5 μM)	105 ± 11 s/30 min	$n=28$	*$p < 0.05$
Metoclopramide (0.4 μM)	158 ± 12s/30 min	$n=29$	*$p < 0.05$
Methoctramine (1 μM)	418.5 ± 15 s/30 min	$n=29$	$p > 0.05$

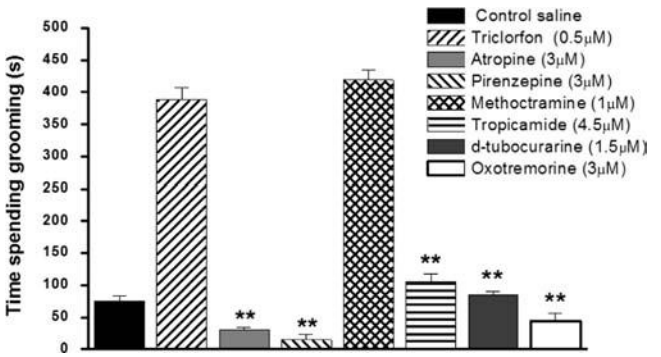

Fig. 4. Effect of different cholinergic modulators on trichlorfon-induced grooming increase in cockroaches. Drugs were injected in the third abdominal segment 10 min before trichlorfon (0.5 μM). The grooming activity was recorded during 30 min and the results expressed as the total time of grooms in seconds. **$p < 0.01$ in comparison to trichlorfon control.

4. Discussion

This work shows the effectiveness of sublethal concentrations of the organophosphate Tn in inducing significative alterations of insect biochemistry, physiology and behavior. The aspects related to the specific mechanisms by which Tn alters insect neurotransmission and its consequent modulation of behavior parameters are outlined below. Trichlorfon is an organophosphate known to block AChE activity and, therefore, to increase cholinergic transmission along the nicotinic, as well the muscarinic pathways as described earlier (Cummings and Ringman 1999). In our experimental conditions, Tn was injected at the cockroach hemocoel and, even at sublethal concentrations, Tn was transported through the hemolymph to act at the CNS of the insect. Based on our toxicological studies on the cockroaches, the sublethal doses of Tn chosen were able to significantly inhibit the brain AChE activity. In addition, there is evidence that organophosphate insecticides last for more than 30 days on the environment in concentrations bearing 30 μg/g (Cheke et al. 2012). This assumption impacts directly in the increase of insect resistance by the persistence of cholinesterase inhibitors such as OPs (Hemingway et al. 2004; Li and Han 2004; Suzuki and Hama 1998), but also review the

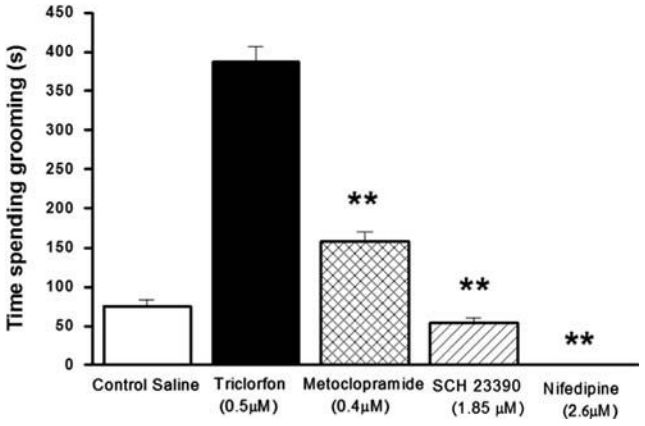

Fig. 5. Inhibition of trichlorfon-induced groomings in cockroaches by dopamine modulators. Drugs were injected in the third abdominal segment 15 min before trichlorfon (0.5 μM). The grooming activity was recorded during 30 min and the results expressed as the total time of grooms in seconds. $^{**}p < 0.01$ in comparison to trichlorfon control.

potential environmental contamination, with inhibition of AChE and behavioral alterations in insects (Guez et al. 2005).

Besides, we found that the observed changes in cockroach behavior induced by sublethal doses of Tn can be associated to a direct influence of the organophosphate on insect cholinergic-dopaminergic co-transmission, probably sharing the same mechanisms observed in mammals (Torres-Altoro et al. 2011). One of these changes was observed in terms of increasing the spontaneous locomotion activity. Monoaminergic systems, and in particular, the dopaminergic systems, are known to profoundly affect motivation and locomotion in insects (Gal and Libersat, 2008). Recently, it has been shown that the monoamine pair dopamine-octopamine plays an important role in cerebral circuits in the induction of hypokinesia and/or hyperkinesias depending on the concentrations (Gal and Libersat, 2008). Such manipulation would affect specific pathways converging, directly or indirectly (for example, via thoracic dorsal unpaired median neurons (Rosenberg et al., 2006), onto thoracic pattern-generating circuits to specifically increase the propensity of walking-related behaviors. Further investigation of these pathways, which represent the link between decisions made in the cerebral ganglia and their execution in the thoracic ganglia, might lead to further understanding of the neuronal basis of motivation and goal-directed actions in insects.

The results presented here also reveal that Tn affects grooming behavior in a complex way, producing quantitative changes. Grooming in insects have the function of cleaning the outer body surface and may have other functions as well, such as courtship behavior, social signaling, displacement activity and de-arousal (Spruijt et al., 1992). In insects, despite the neural center involved in grooming behavior is still unknown, it has been already demonstrated that the main neurotransmitter associated with this response is dopamine (Weisler-Eicheler et al., 1999).

In our experimental conditions, application of the selective D_1-inhibitor SCH23390 significantly contributed to the Tn-induced grooming increase, clearly demonstrating that the DA-D_1 receptor class is involved in controlling this kind of behavior in insects (Libersat 2003; Mustard et al. 2010). However, the effectiveness of methoclopramide in preventing the Tn-induced increase of grooming, suggests a poor selectivity of this drug over insect DA-D_2 receptors and the concomitant blockage of insect D_1 receptors (Degen et al. 2000). In addition, it is worthwhile to notice the influence of nifedipine in counteracting with the increase of the time spent with grooming induced by Tn, as expected. We speculate that this raise in the grooming time could be related to the recent discovery that the activation of D_1-like receptors activate and open calcium channels at the presynaptic regions of dopaminergic neurons of immature rats (Momiyama and Fukazawa 2007).

It is presumed that a behavior so complex as grooming may be mediated by a region somewhere in the brain of the insects (Libersat et al. 2009). To date, the insect nervous system consists of a central nervous system (CNS), formed by a cephalic supra, sub and circumesophageal ganglion mass or brain, and a peripheral nervous system (PNS), which includes the stomatogastric nervous system (Osboure, 1996). Klemm (1976) reported that the stomatogastric nervous system contains a frontal ganglion, which is connected to the brain via two frontal nerves and the *nervus connectivus*, and to the hypocerebral ganglion by a single recurrent nerve. The latter is linked to the corpora cardiaca and to the two ventricular ganglia that send nerves to the gut. The frontal and hypocerebral ganglia of the stomatogastric nervous system consist of approximately 200 neurons using a variety of different neurotransmitters (Klemm et al., 1986). Therefore, a number of projections of this neuronal system provide acetylcholine through axons of cholinergic neurons whose somata are located in other nuclei (Alcantara et al., 2003; Aosaki et al., 2010). Thus, ACh is found in very high levels within the insect nervous tissue (Le Corronc and Hue, 1993), and it appears to be the major neurotransmitter used by the insect sensory neurons (Pitman, Fleming, 1985). In addition, ACh is known to be an effective target site for several insecticides (Corbett et al., 1984; Lummis and Sattelle, 1985).

Two major types of ACh receptors have been characterized in insects: nicotinic acetylcholine receptors (nAChrs) and muscarinic acetylcholine receptors (mAChrs) (Le Corronc and Hue, 1993). The distribution of nAChrs in insect nervous system is quite complex and numerous studies have revealed the pharmacological properties of these receptors on synapse-free cell bodies of neurons as well as on the synapse–neuropile complex (Suter and Usherwood, 1985; Breer and Sattelle, 1987; Thany and Tricoire-Leignel, 2011). It is assumed that at synaptic level, nAChRs are associated with postsynaptic mediated excitation and membrane depolarization (Mac Dermott et al., 1999; Albuquerque et al., 2009), while at presynaptic sites nicotinic activation could produce either excitation or inhibition indirectly through the release of endogenous transmitters or modulators (Fu and Liu, 1996; Thainy and Tricoire-Leignel, 2011). In insects it is found that the presynaptic muscarinic receptors act as autoreceptors, regulating the release of ACh (Le Corronc et al., 1991; Leitch and Pitman, 1995; Hue et al., 1989) while postsynaptic muscarinic receptors reduced the giant fiber spike threshold (Le Corronc and Hue, 1991). The release-enhancing presynaptic muscarinic and nicotinic receptors co-exist and interact on dopaminergic nerve endings of rat CNS (Grilli et al., 2008).

We suggest that the inactivation of AChE by sublethal doses of Tn increases the levels of ACh at the insect nervous system, overstimulating both nicotinic (nAChR) and muscarinic (mAChR) receptors (Osboure, 1996), increasing the number of action potentials towards the insect brain. In this scenario, the ACh released after stimulation of the sensory neuron would activate cholinergic muscarinic receptors (M_1 –M_1, M_3, M_5– or M_2 –M_2, M_4– classes) (Aosaki et al., 2010; Li et al., 2009), or nicotinic (N_1-N_2) (Libersat, 2003) at somata of dopaminergic neurons, which would in turn modulate the release of dopamine onto the sensory afferent terminal (Torres-Altoro et al., 2011). Indeed, nicotine exhibits differential dose-dependent actions on both insect and mammalian presynaptic nAChRs, stimulating dopamine release and activating dopamine D_1- and D_2-dependent signaling pathways in central nervous system (Torres-Altoro et al., 2011). Activation of presynaptic muscarinic receptors at the cercal afferent-giant interneuron synapses in the cockroach, *Periplaneta americana*

(Le Corronc and Hue, 1993), and at the planta hair afferent-proleg motoneuron synapses in the tobacco hornworm, *Manduca sexta* (Trimmer and Weeks, 1989, 1993) results in a reduction in the spike threshold and an increase in the excitability of the post-synaptic neuron.

Our results are consistent with the hypothesis that neurons releasing dopamine are in fact modulated by cholinergic auto-receptors (Alcantara et al. 2003; Grilli et al., 2009; Threlfell et al., 2010). Application of general muscarinic modulators such as atropine, pirenzepine, oxotromorine and tropicamide, prior to Tn showed that pirenzepine > atropine > oxotremorine > tropicamide were effective in reducing the time spent with grooming, suggesting that the M_1AChR receptors are prevalent for this signal modulation (Aosaki et al., 2011). Furthermore, methoctramine failed to alter Trichlorfon-induced grooming activity, excluding, at least in our experimental conditions, the effectiveness of M_2-M_3AChR autoreceptors for the dopamine modulatory mechanisms (Osboure, 1996; Judge and Leitch, 1999; Aosaki et al., 2011). The significant decrease in Tn-induced increase of grooming caused by *d*-tubocurarine also suggests that nicotinic cross-talking is possibly involved in the dopaminergic pathway at the insect central nervous systems (Salvaterra, Foders, 1979, Grilli et al., 2009).

Sublethal effects of organophosphate insecticides on insect behavior are important to be determined. Most of insecticides affect one of the five biological systems in insects. These include the nervous system, the production of energy, the recycling of cuticle, the endocrine system and the water balance. The nervous system is far the most important, since it can lead to unpredictable changes in behavior such as feeding patterns (Nicolaus and Lee 1999), reproduction (Delpuech et al. 2005), foraging (Guez et al. 2005), migration and thermoregulation (Grue et al. 1997), among others. The results point out that the persistence of insecticides in the environment should not be an ignored issue, and that these sublethal doses are amenable to cause significant influences on the nervous system of animals (e.g. changing behavioral patterns). Further studies on Tn related to sublethal effects on *P. pallida* should be carried out, especially those studying the influence of the acylpeptide hydrolase on the expression of behavioral changes (Richards et al., 2000). Studies on the molecular biology associated to electrophysiological approaches will be also necessary in order to improve the knowledge about cholinergic/dopaminergic cross-talking in insects.

5. Conclusions

The results suggest that the environmental persistence of insecticides should not be an ignored issue, and that sublethal doses of organophosphate insecticides are amenable to cause significant influences on the nervous system of animals (e.g. changing behavioral patterns). They also indicated that these cholinergic modulators directly modulate dopaminergic signaling in insects, influencing locomotor activity. These behavioral alterations would severely impact the insect orientation and locomotion, and thus have the potential for serious ecological consequences including other beneficial insects through trophic transfer.

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Linhas de pesquisa
Entomotoxicidade de compostos vegetais

Linhas de pesquisa

1. Entomotoxicidade de compostos vegetais

Objetivo: Atividade farmacológica de extratos vegetais em modelos experimentais de insetos..
Grande área: Ciências Biológicas
Grande Área: Ciências Biológicas / Área: Morfologia / Subárea: Histologia.
Palavras-chave: neurotoxicidade.

2. Atividade entomotóxica de enzimas e peptídeos

Objetivo: Avaliar o potencial inseticida de ureases e peptídeos derivados, visando esclarecer mecanismos de ação relacionados a esta atividade..
Grande área: Ciências Biológicas
Grande Área: Ciências Biológicas / Área: Bioquímica / Subárea: Biologia Molecular.
Palavras-chave: bioinseticidas; Sistema Nervoso Central; Junção Neuromuscular.

Projetos de pesquisa

2013 - Atual Efeitos da entomotoxina jaburetox e derivados sobre o sistema neurolocomotor de insetos

Descrição: Este projeto tem por objetivo realizar a caracterização da atividade entomotóxica de peptídeos derivados de ureases de canavalia ensiformis..
Situação: Em andamento; Natureza: Pesquisa.

Integrantes: Thiago Carrazoni de Freitas - Integrante / Marines de Ávila Heberle - Integrante / Marina Schumacher Defferrari - Integrante / Célia Regina Carlini - Coordenador / Chárist on André Dal Belo - Integrante.

2010 - Atual Atividade inseticida do extrato metanólico de Araucaria angustifolia

Descrição: Avaliar por meio de ensaios fitoquímicos e farmacológicos o potencial inseticida do extrato metanólico das folhas de Araucaria angustifolia em modelo experimental de barata..
Situação: Em andamento; Natureza: Pesquisa.

Integrantes: Thiago Carrazoni de Freitas - Coordenador / Chárist on - Integrante.
Financiador(es): Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul - Bolsa.

2009 - 2010 Atividade antiofidica do oleo essencial de Lafoensia pacari

Descrição: Avaliar o potencial anti-ofídico do óleo essencial de Lafoensia pacari em camundongos.
Situação: Desativado; Natureza: Pesquisa.

Integrantes: Thiago Carrazoni de Freitas - Coordenador.

2008 - 2009 HYPOKINETIC EFFECTS-INDUCED BY Rhinella icterica VENOM ON COCKROACH NEUROLOCOMOTOR SYSTEM

Descrição: Background: Rhinella icterica is a common Brazilian toad specimen that occurs from forests to open areas, as the ?Cerrado? region. The knowledge about its venom pharmacology may lead to the discovery of novel bioactive compounds with potential medical use. In this work we assayed the venom of R. icterica on insect motor behavior parameters..
Situação: Concluído; Natureza: Pesquisa.
Alunos envolvidos: Graduação: (3) .

Integrantes: Thiago Carrazoni de Freitas - Coordenador / Magali - Integrante / Chárist on - Integrante / Neverton - Integrante / Marilia - Integrante / Lúcia - Integrante.
Financiador(es): Universidade Federal do Pampa - Bolsa.

2007 - 2008 Caracterização dos residuos solidos da cidade de São Gabriel - RS

Descrição: Caracterização e elaboração de um plano de gerenciamento dos residuos solidos da ciadade de São Gabriel - RS, Brasil..
Situação: Concluído; Natureza: Pesquisa.

Integrantes: Thiago Carrazoni de Freitas - Coordenador.

Áreas de atuação

1. Grande área: Ciências Biológicas / Área: Fisiologia / Subárea: Fisiologia de Órgãos e Sistemas/Especialidade: Neurofisiologia.

2. Grande área: Ciências Biológicas / Área: Bioquímica / Subárea: Biologia Molecular.

3. Grande área: Ciências Biológicas / Área: Biofísica / Subárea: Biofísica Celular.

4. Grande área: Ciências Biológicas / Área: Farmacologia / Subárea: Toxicologia.

Idiomas

Inglês	Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Espanhol	Compreende Bem, Fala Razoavelmente, Lê Bem, Escreve Pouco.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica

1. **CARRAZONI, THIAGO**; DE AVILA HEBERLE, MARINES ; PERIN, ANA PAULA ARTUSI ; ZANATTA, ANA PAULA ; RODRIGUES, POLYANA VELOSO ; DOS SANTOS, FABIOLA DUARTE MACHADO ; DE ALMEIDA, CARLOS GABRIEL MOREIRA ; VAZ BREDA, RICARDO ; DOS SANTOS, DOUGLAS SILVA ; PINTO, PAULO MARCOS ; DA COSTA, JADERSON COSTA ; CARLINI, CELIA REGINA ; DAL BELO, CHÁRISTON ANDRÉ . Central and peripheral neurotoxicity induced by the Jack Bean Urease (JBU) in Nauphoeta cinerea cockroaches. Toxicology (Amsterdam) JCR, v. 368-369, p. 162-171, 2016.

2. DOS SANTOS, DOUGLAS SILVA ; CARVALHO, EVELISE LEIS ; DE LIMA, JEFERSON CAMARGO ; BREDA, RICARDO VAZ ; OLIVEIRA, RAQUEL SOARES ; **DE FREITAS, THIAGO CARRAZONI** ; SALAMONI, SIMONE DENISE ; DOMINGUES, MICHELLE FLORES ; PIOVESAN, ANGELA REGINA ; BOLDO, JULIANO TOMAZZONI ; DE ASSIS, DÊNIS REIS ; DA COSTA, JADERSON COSTA ; DAL BELO, CHÁRISTON ANDRÉ ; PINTO, PAULO MARCOS . Bothriurus bonariensis scorpion venom activates voltage-dependent sodium channels in insect and mammalian nervous systems. Chemico-Biological Interactions (Print) JCR, v. 258, p. 1-9, 2016.

3. DAIANE STU¨RMER, GRAZIELE ; **DE FREITAS, THIAGO CARRAZONI** ; DE AVILA HEBERLE, MARINES ; DE ASSIS, DÊNIS REIS ; VINADÉ, LÚCIA ; BATISTA PEREIRA, ANTÔNIO ; LUIS FRANCO, JEFERSON ; ANDRÉ DAL BELO, CHÁRISTON . Modulation of dopaminergic neurotransmission induced by sublethal Doses of the organophosphate trichlorfon in cockroaches. Ecotoxicology and Environmental Safety JCR, v. 109, p. 56-62, 2014.

Citações: WEB OF SCIENCE 1 | SCOPUS 3

Apresentações de Trabalho

1. **DE FREITAS, THIAGO CARRAZONI**; LANGE, A. B. ; CARLINI, C. R. ; ORCHARD, I. . THE EFFECTS OF THE ENTOMOTOXIN JACKBEAN UREASE ON INSECT NEUROMUSCULAR SYSTEM. 2015. (Apresentação de Trabalho/Conferência ou palestra).

2. Freitas ; Pesamosca ; Posser. T ; Hyslop. S ; Franco. J ; Dal Belo ; **Freitas** . INSECTICIDAL ACTIVITY OF Rhinella icterica TOAD VENOM. 2010. (Apresentação de Trabalho/Congresso).

3. LUCHO, A. P. ; **Freitas, TC** ; HEBERLE, M. A. ; PERDOMO, J. D. ; CORREA, M. S. ; Dal Belo . Avaliação da Atividade Bloqueadora Neuromuscular do Extrato Metanólico de Araucaria angustifolia em Baratas. 2010. (Apresentação de Trabalho/Simpósio).

4. **Freitas, TC**; LUCHO, A. P. ; Franco. J ; Dal Belo ; Silva, P. G . Manipulação do Comportamento Psicomotor Induzido Pelo Extrato Metanólico de Araucaria angustifolia Em Insetos. 2010. (Apresentação de Trabalho/Simpósio)

(Apresentação de Trabalho/Simpósio).

5. Pesamosca ; **Freitas, TC** ; Franco. J ; Dal Belo . Preliminary Biochemical and Pharmacological Characterizations of Rhinella icterica Toad venom. 2010. (Apresentação de Trabalho/Congresso).

6. **Freitas**; Pesamosca ; Vaz ; Hartmann ; Vinadé ; Dal Belo . HYPOKINETIC EFFECTS-INDUCED BY Rhinella icterica VENOM ON COCKROACH NEUROLOCOMOTOR SYSTEM. 2009. (Apresentação de Trabalho/Congresso).

Eventos

Participação em eventos, congressos, exposições e feiras

1. Insect Biotech Conference. THE EFFECTS OF THE ENTOMOTOXIN JACKBEAN UREASE ON INSECT NEUROMUSCULAR SYSTEM. 2015. (Congresso).

2. 42º Congresso Brasileiro de Farmacologia e Terapêutica Experimental. Preliminary Biochemical and Pharmacological Characterizations of Rhinella icterica Toad venom. 2010. (Congresso).

3. II Salão Internacional de Ensino, Pesquisa e Extensão.Avaliação Fitoquimica Preliminar do Extrato Etanólico de Araucaria angustifolia. 2010. (Encontro).

4. II Salão Internacional de Ensino, Pesquisa e Extensão.Avaliação da Atividade Bloqueadora Neuromuscular do Extrato Metanólico de Araucaria angustifolia em Baratas. 2010. (Encontro).

5. II Salão Internacional de Ensino Pesquisa e Extenção. Manipulação do Comportamento Psicomotor Induzido Pelo Exrato Metanólico de Araucaria angustifolia em Insetos. 2010. (Congresso).

6. XI Congresso da Sociedade Brasileira de Toxinologia. INSECTICIDAL ACTIVITY OF Rhinella icterica TOAD VENOM. 2010. (Congresso).

7. Biofórum Especial: Simpósio de Imonologia. 2009. (Simpósio).

8. Fórum Gabrielense de Inclusão. 2009. (Seminário).

9. I Fórum Ambiental da Universidade Federal do Pampa. 2009. (Simpósio).

10. I Salão Internacional Integrado de Ensino, Pesquisa e Extensão, IX Mostra Científica Internacional, Salão Internacional de Iniciação Científica, II Salão Internacional de Extensão e I Salão Internacional de ensino. 2009. (Encontro).

11. I Semana do Meio Ambiente. 2009. (Encontro).

12. World Conference on Toxinology. HYPOKINETIC EFFECTS-INDUCED BY Rhinella icterica VENOM ON COCKROACH NEUROLOCOMOTOR SYSTEM. 2009. (Congresso).

13. III Worshop em Namociências. 2008. (Oficina).

14. VIII Salão de Iniciação Científica - Ed. Internacional, VIII Mostra Científica - Ed. Internacional e I Feira de Extensão - Ed. Internacional.. 2008. (Encontro).

15. I Semana Acadêmica Integrada. 2007. (Encontro).

Organização de eventos, congressos, exposições e feiras

1. **Freitas, TC**. I Semana Acadêmina Integrada - Unipampa. 2007. (Exposição).

Inovação

Projetos de pesquisa

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